

UNIVERSITY OF EDINBURGH

"An investigation into the actions of certain anthelmintics".

Thesis presented for the Degree of Doctor of Philosophy

by

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Part of Section II, Part II, has been  
accepted for publication in the Archives internationales  
de pharmacodynamie.

## General Introduction

In recent years, methods of livestock production in many parts of the world have become more intensive. This has meant that pastures have become more heavily contaminated, and, consequently, losses from parasitic infestations have become more serious. It is now recognised that even sub-clinical infestations of internal parasites in young stock may result in heavy losses by retarding weight gains and by lowering the resistance of the animals to other infections. Up to the present, higher standards of animal husbandry and the discovery and application of drugs for the treatment of parasitic infestations have only improved the position to a limited extent. Unfortunately most of the anthelmintic drugs in use, especially in sheep and cattle, are toxic, expensive or unreliable in action, and they are often difficult to administer. They are all to some extent selective in action so that the administration of a single drug seldom relieves an animal of its entire worm burden. The aim of this work was to investigate some of the actions and properties of existing anthelmintics, and thus to obtain information which could be applied to the search for more satisfactory drugs for the treatment of helminthiasis.



## SECTION I

### Preliminary Experiments

#### Introduction

It was decided to make an attempt to keep some parasitic worms alive in vitro so that their responses to drugs could be studied directly. The type of experiment envisaged was a long-term one so that slow changes in the helminth caused by repeated low doses of a drug could be studied. The possibility of keeping parasitic worms alive not only in an incubator but inside the intestine of ponies provided with various intestinal fistulae was considered. So far as is known, no attempts have been made previously to keep adult nematodes alive in this way.

In vitro studies on anthelmintic drugs possess the advantage that the possibility of the drug being altered chemically in the body of the host is excluded. On the other hand, in vitro techniques for screening drugs for anthelmintic activity have the disadvantage that compounds which are successful may prove to be useless in vivo either because they are toxic to the host, or rapidly and completely absorbed before reaching the worms, or altered in the alimentary tract to ineffective compounds.

Early /

Early attempts to screen numbers of compounds for anthelmintic activity in vitro were carried out on annelid worms. (Trendelenburg, 1916). Unfortunately, results from these tests were not applicable to parasitic helminths which have a much less permeable cuticle.

Lamson and Brown (1936) tested numbers of compounds for anthelmintic activity on whole Ascaris lumbricoides in vitro.

Baldwin (1943) used segments of Ascaris tied off at each end and suspended in a suitable fluid, and recorded the movements made by the preparations when different drugs were added to the medium. This technique has been used by a number of workers (Mackie 1953; Mackie, Stewart, Cutler and Misra, 1955), but only drugs which permeate the nematode cuticle and which act on the neuro-muscular apparatus of the worm can be expected to affect the movements of this preparation. The method is only really suitable for the comparison of the activity of a number of closely related compounds.

Continuing the search for preparations suitable for screening numbers of compounds in vitro, Leiper (1952) used Vinegar eel-worms (Anguillula aceti); while Rogers (1944) used the perfused isolated worm-infected intestine.

Levine /

Levine and Ivens (1953, 1954) tested numbers of compounds on the development of free-living stages of horse strongyles in the faeces. All these tests suffered from the disadvantage that drugs which were active in them were not necessarily effective in vivo.

All anthelmintics are specific to a certain extent and all tests on a single worm species have the disadvantage that results from one species cannot be expected to apply to others, particularly to nematodes which are not closely related or which do not belong to the same part of the alimentary tract.

Attempts to keep nematodes alive in vitro

Materials and Methods

Because of the possibility of maintaining worms alive inside fistulated ponies, it was decided to obtain parasitic worms from horses. The worms were obtained from newly killed horses in the slaughter-house. They were removed directly from the intestine and transported to the laboratory in wide-mouthed vacuum flasks filled with Tyrode's solution at 37°C. Nematodes from other species were obtained occasionally, and treated in a similar way.

Ascaris equorum: Each of these specimens was put into a glass jar fitted with a ground glass stopper. This was filled to within about half an inch from the top with Tyrode's solution and kept in an incubator at 37°C. The total volume of the solution was about 225 ml. Various modifications were made to the Tyrode's solution in order to find the best medium for the worms.

- 1) On some occasions a solution without glucose but otherwise identical in composition to Tyrode's solution was used. This solution is afterwards referred to as medium B.
- 2) In some experiments penicillin was added to prevent bacterial growth (0.04 units/ml.).
- 3) In an attempt to provide a relatively anaerobic medium for the Ascaris the Tyrode was covered with a layer of liquid paraffin.
- 4) /



- 4) Freshly obtained horse intestine contents were centrifuged and the supernatant kept in a boiling water-bath for half an hour to sterilise it. The pH of this material before and after sterilisation measured 6.9 and 7.2 respectively. 40 ml. of the supernatant was substituted for an equal volume of Tyrode. It was thought to be possible that some nutrient material normally available to the helminths would be present in this material. If helminths survived longer in this medium than in Tyrode's solution a systematic search for the essential constituent could have been undertaken.

The solutions were changed at frequent intervals, partly to prevent excessive bacterial growth, and partly because the worms excrete volatile fatty acids, causing a fall in pH of the medium. Helminths were kept in the same solutions for varying lengths of time and the state of acidity of the medium then measured using a Marconi pH meter.

Oxyuris equi: A number of these were found on one occasion only. Most of them were kept in the incubator at 37°C. Five were put into normal Tyrode and five into medium B.

Strongylus equinus: A number of adults were kept in the incubator in Tyrode's solution at 37°C, using smaller containers than those for Ascaris equorum. Some Strongylus equinus larvae were found in the pancreas of a newly killed horse. Some of these were kept in the incubator in the same way as the adult specimens.

Haemonchus contortus (from sheep): Some of these were kept at 37°C in jars full of normal saline, and others in Tyrode's solution.



Ascaris lumbricoides var suis: Most of these were kept in medium B. Some were kept in a mixture of Tyrode and the supernatant from centrifuged horse intestine contents. Others were kept in this mixture with added penicillin. The pH of these media was taken after twenty-four hours.

Toxascaris leonina (from dogs): These specimens were kept in medium B. The pH of the medium was taken after forty-eight hours.

Composition of Tyrode's solution

Sodium chloride	0.8%
Potassium chloride	0.02%
Calcium chloride	0.02%
Magnesium chloride	0.01%
Sodium bicarbonate	0.01%
Sodium dihydrogen phosphate	0.005%
Glucose	0.1%
Water	99.035%

Results /

# Results

The results of these experiments are given in Table A.

TABLE A

Duration of Survival of Nematodes incubated at 37° C.

SPECIES	No. of Worms	MEDIUM WHILE IN INCUBATOR	DURATION OF SURVIVAL
<i>Ascaris equorum</i>	14	Tyrode's solution	10-14 days
<i>Ascaris equorum</i>	10	Medium B	10-15 days
<i>Ascaris equorum</i>	5	Tyrode + penicillin	9-14 days
<i>Ascaris equorum</i>	2	Surface of Tyrode covered with layer of liquid paraffin	24 hours
<i>Ascaris equorum</i>	5	Tyrode + horse intestine contents	3-5 days
<i>Oxyuris equi</i>	5	Tyrode's solution	5-12 hours
<i>Oxyuris equi</i>	5	Medium B	4-12 hours
<i>Strongylus equinus</i> (adults)	7	Tyrode's solution	18 hours
<i>S. equinus</i> (larvae)	14	Tyrode's solution	4 days
<i>Haemonchus contortus</i>	35	Tyrode's solution	18-20 hours
<i>Haemonchus contortus</i>	35	0.9% Sodium chloride solution	18-20 hours
<i>Ascaris lumbricoides</i>	6	Tyrode's solution	12-21 days
<i>Ascaris lumbricoides</i>	15	Medium B	12-19 days
<i>Ascaris lumbricoides</i>	5	Tyrode + horse intestine contents	3-4 days
<i>Ascaris lumbricoides</i>	5	Tyrode + horse intestine contents + penicillin	3-4 days
<i>Toxascaris leonina</i>	4	Medium B	3-5 days

Preliminary experiments had shown that survival of the parasites was longest when the medium was changed at least once a day. No difference in survival time between male and female worms was apparent in any of these species.

The adult female Ascaris equorum and Ascaris lumbricoides shed quantities of eggs daily.

The pH readings of solutions in which the parasites had been incubated are contained in Table B.

TABLE B.

The effect of the presence of Ascaris on the pH of the medium.

Species	Duration of incubation (hours)	Medium	pH before incubation	pH after incubation	Number of experiments
<u>Ascaris equorum</u>	24	Tyrode's soln.	8.1	6.9	Average of 4 experiments
"	48	" "	8.1	6.4	1 experiment
<u>Ascaris lumbricoides</u>	24	" "	8.1	6.8	Average of 4 experiments
" "	24	Tyrode's + intestinal contents	7.8	6.1	Average of 2 experiments
" "	24	Tyrode's + intestinal contents + penicillin	7.8	6.1	Average of 2 experiments
<u>Toxascaris leonina</u>	48	Medium B	8.1	7.2	1 experiment

### Conclusions

From the results of the experiments with Ascaris equorum and Ascaris lumbricoides it could be seen that:

- 1) The presence or absence of glucose did not appear to make much difference to the survival of the worms.
- 2) The addition of penicillin made no appreciable difference if the solutions were changed daily.
- 3) Covering the Tyrode's solution with liquid paraffin was not a success. The Ascaris were dead within twenty-four hours.
- 4) The addition of boiled horse intestinal contents with or without added penicillin resulted in premature death of the Ascaris. All were dead within three to five days.

With the exception of Ascaris equorum, Ascaris lumbricoides and the larvae of Strongylus equinus none of the parasites survived for more than a few hours in the incubator. Responses of the smaller nematodes to drugs while in such an obviously unfavourable environment would probably be very abnormal.



## Culture of Ascaris Equorum Eggs

### Methods

The adult female Ascaris equorum shed quantities of eggs while in the incubator; these were collected and examined under the microscope. Cell division appeared to be taking place normally in the majority of the eggs. The eggs of Ascaris equorum are said to be infective in ten days or longer depending on the temperature. (Monnig, 1947). Some of the eggs were centrifuged with boiled Tyrode's solution and left on damp filter paper in a petri dish for ten days at room temperature (about 18°C.) then at 26°C. for a further day. The larvae could then be seen under the microscope. They were motile at this stage.

It was hoped that it would be possible to digest away the thick albuminous outer layer of the eggs and so obtain the larvae for test material.

Ileum contents were obtained from a pony with a Bieble loop, centrifuged, and the eggs incubated in the supernatant at 38°C. Other fully embryonated eggs were incubated with pepsin and trypsin solutions of appropriate pH. These were made up as follows: 1% solutions of pepsin and trypsin were made up in distilled water.

a) /



- a) 1 ml. 1% pepsin and 5 mls. 0.1 N HCl. pH = 1

(This solution digested a piece of fibrin  
in 15-20 minutes at 38°C.

- b) 5 ml. 1% trypsin and 5 mls. 0.3% NaHCO<sub>3</sub> pH = 8

(This solution digested a piece of fibrin  
in 1½ hours at 38°C.

The eggs were removed at intervals for microscopic  
examination.

Although adult Ascaris equorum will not infect  
another species, hatching of the eggs and migration of the  
larvae through the lungs is said to take place in many  
vertebrates. (Monnig, 1947). Attempts were made to obtain  
the larvae by the administration of fully embryonated eggs  
to mice. A week after being given the eggs the mice were  
killed. The Baermann technique was employed in an attempt  
to recover larvae. (Morgan and Hawkins, 1949). The livers  
and lungs were cut into small pieces and put into a muslin  
bag which was suspended in a filter funnel. Water at 40°C.  
was poured through slowly and collected in a beaker. The  
contents of the beaker were examined for larvae.

## Results

TABLE C.

Effect of enzymes on hatching of fully embryonated Ascaris equorum eggs.

Number of eggs	Treatment	Result
100-150	Incubated at 38°C with centrifuged ileum contents	None were hatched within 48 hours
50-60	Incubated at 38°C with pepsin	None were hatched within 48 hours
50-60	Incubated at 38°C with trypsin	None were hatched within 48 hours

When 20-30 eggs were suspended in water and given to each of eight mice by stomach tube, no larvae were found when the chopped livers and lungs of the mice were subjected to the Baermann technique.

The larvae could be released apparently undamaged from the albuminous outer layer by gently pressing the eggs between two glass slides, but they did not survive in the incubator for more than an hour or two after this treatment.

Conclusion. Incubation of fully embryonated Ascaris eggs with horse ileum contents and in pepsin and trypsin solutions did not release the larvae from the albuminous outer layer of the eggs.

Administration of the eggs to mice by stomach tube in the hope that they would hatch in the intestines of these animals and the larvae migrate to the liver or lungs did not meet with success.

Attempts to maintain nematodes alive in the intestine  
of fistulated ponies.

Methods

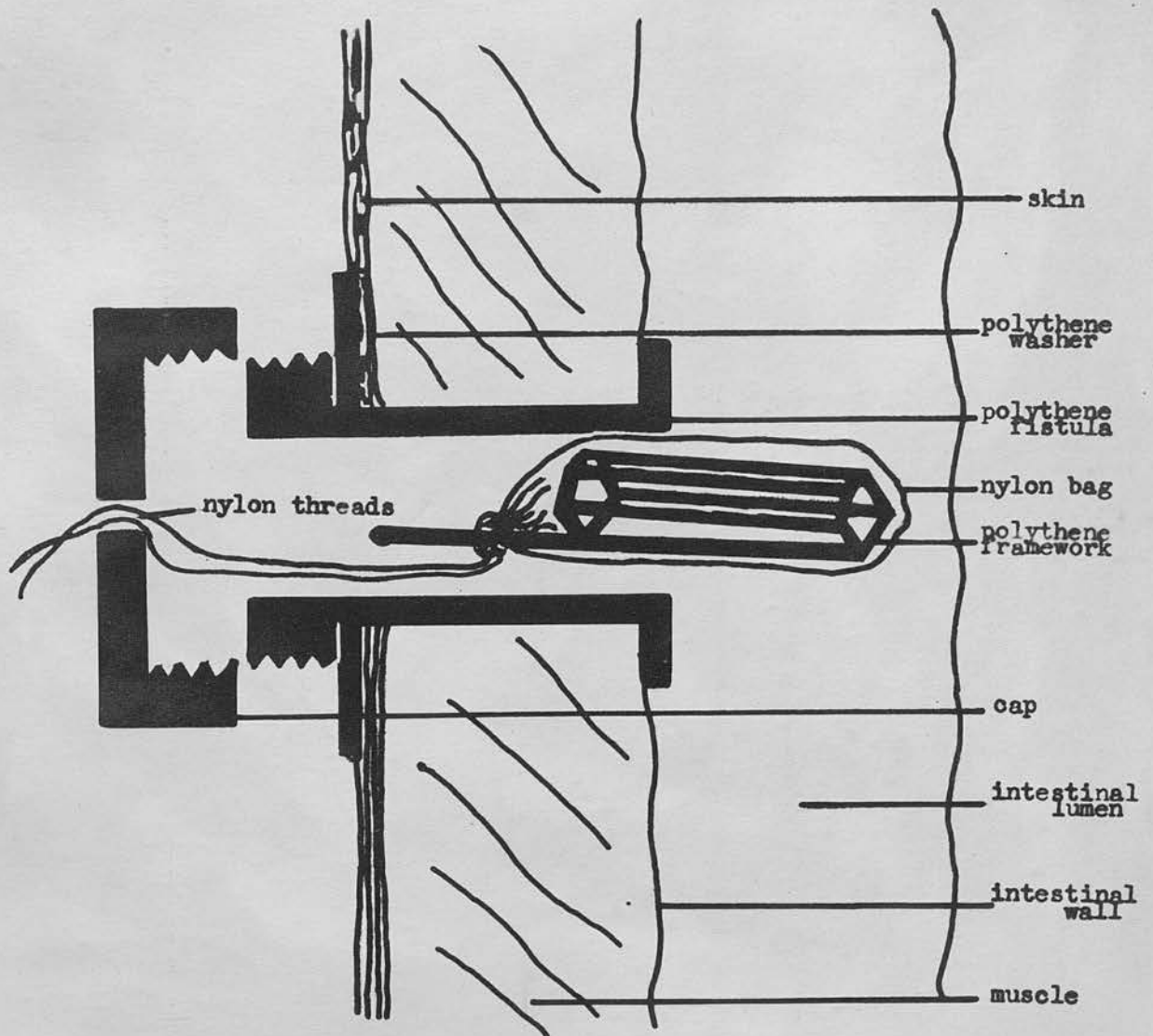
When some of the smaller nematodes were obtained from horses, these were put into very small bags made of fine mesh nylon material and these were gently pushed down a fistula into the colon of the pony. The fistula cap was then replaced. Each bag with its contents could be removed when necessary by pulling on strong nylon threads which fastened the necks of the bags and led to the exterior.

After the first few experiments small polythene supports were inserted inside the bags to prevent the worms from being squashed when the bags were pushed down the fistulae.

These supports consisted of two pentagonal frames joined at the corners by five polythene rods. Each side of the pentagon measured approximately 9 mm; four of the rods were 45 mm. long. The fifth rod was extended 25 mm. beyond the others so that the projecting end could be held in the neck of the nylon bag. (See illustration, Page 14a).

Oxyuris equi, Strongylus equinus adults, and Strongylus equinus larvae from the pancreas of the horse were treated in this way.

Diagram showing intestinal fistula and nylon bag in position.





Results.

TABLE D.

Survival time of nematodes kept in the intestine of fistulated ponies

SPECIES	NUMBER OF WORMS	SURVIVAL TIME (HOURS)	
		Greater than	Less than
<i>Oxyuris equi</i> " "	4	22	25
	2 (using polythene support)	23	25
<i>Strongylus equinus</i> (adults) " " "	2	16	18
	3 (using polythene support)	16	18
<i>Strongylus equinus</i> (larvae)	3 (using polythene support)	16	18

The survival times given are approximate, depending on the time intervals between inspection of the worms.

The insertion of the polythene supports into the nylon bags did not prolong the lives of the nematodes.

Conclusions. Attempts to keep nematodes alive in nylon bags in the intestine of a fistulated pony were not successful, although *Oxyuris equi* survived under these conditions rather longer than in the incubator.

*Strongylus equinus* adults survived for as long a time in the intestine as they did in the incubator, but *S. equinus* larvae died much more quickly in the former than in the latter environment.



### Discussion

It was apparent that only the Ascaris equorum and lumbricoides survived long enough to be suitable for in vitro studies. Other worms from horses were difficult to obtain because most of the slaughtered horses had been entirely stable fed.

The failure of pepsin and trypsin to hydrolyse the outer layer of the Ascaris eggs (page 13) may well be a result of the specificity of these enzymes. Pepsin hydrolyses only peptide bonds which are adjacent to an aromatic amino acid, (Baker, 1951) while trypsin acts on peptide links involving either lysine or arginine (Hoffman and Bergmann, 1941). Presumably the protein of which the egg-shell is composed does not possess these linkages. Bird and Rogers (1956) found that these enzymes failed to hydrolyse the larval sheaths of Haemonchus contortus, Trichostrongylus spp. and Nippostrongylus muris.

It was disappointing that attempts to keep smaller helminths alive in the fistulated pony (page 15) were unsuccessful.

Although it was not surprising that the Strongylus equinus larvae from the pancreas failed to survive for long in the intestine, it was difficult to understand why the other specimens were not maintained in a healthy condition.

If /

If these experiments had been successful they would have provided a unique opportunity for the study of these species while in their normal environment.

When it became clear that only Ascaris spp. were able to survive for more than a few hours in vitro it was decided not to pursue the in vitro investigations further. This was partly because Ascaris spp. have already been extensively studied in vitro by other workers, but chiefly because it had been decided by this time that the most interesting anthelmintic to study would be phenothiazine. Even in vivo phenothiazine is relatively ineffective against Ascaris spp. and it has been shown to have no effect on Ascaris lumbricoides in vitro (Collier, 1940; De Eds and Thomas, 1941). Phenothiazine has no effect on Baldwin's preparation, and does not appear to have much effect on any nematodes in vitro. Lazarus and Rogers (1950, 1951) have shown that it is taken up by nematodes in vitro, but more slowly than in vivo.

Taylor and Sanderson (1940) found that saturated and half saturated solutions of phenothiazine in physiological saline had some deleterious effect on Trichostrongylus spp. in vitro, but their results seem a little inconclusive as all the treated worms did not die for sixty-six hours.

## SECTION II

### STUDIES ON PHENOTHIAZINE

#### Introduction

Phenothiazine is one of the more successful and perhaps the most interesting of the anthelmintic drugs in use at present. It was first prepared by Bernthsen in 1883; for many years it was only of interest because it was the parent substance of a number of dyes, notably methylene blue and Lauth's violet. Biologists first became interested in it when Campbell, Sullivan, Smith and Haller (1934) discovered that it had insecticidal properties. Its uses, however, were limited because when it was applied as a dust or spray it gave rise to photosensitisation of human skin. (De Eds, Wilson and Thomas, 1940). Its remarkable anthelmintic properties first came to light in 1938 when Harwood, Jerstad and Swanson found that it expelled ascarids and nodular worms from pigs. This discovery was followed by trials with phenothiazine in other infected species, and extensive use of the drug has proved its value, in particular against various nematodes of sheep (Swales, 1940), stomach and nodular worms in cattle (Swanson and Batte, 1952) and small strongyles in horses (Gibson, 1950). It is also effective /

effective in poultry for the eradication of Heterakis gallinae, and slightly less so for Ascaridia galli.

(Turk, 1958). It may be administered as a drench, in capsules or tablets. Reflex stimulation of the oesophageal groove is unnecessary as the drug is equally effective when given into the rumen or the abomasum. There is evidence that it is more effective when given as a single dose than when given as several divided doses. (Steward, 1955; Douglas, Baker and Longhurst, 1957).

Considering the widespread use of phenothiazine and the large doses of it which are frequently given without ill-effects, it cannot be considered a really toxic drug. Nevertheless, numerous cases of phenothiazine poisoning have occurred. In general, young animals are more susceptible than adults to its toxic effects, and a wide variation in susceptibility between individuals of one species is characteristic.

Phenothiazine is peculiar in that the occurrence of toxic effects seldom seems to be related to the size of the dose. Toxic manifestations have sometimes occurred in a group of animals after therapeutic doses, whilst individuals have survived enormous amounts of the drug. (Rogers, Brooks and Higgs, 1957). It is possible that diet plays a part by influencing the degree of absorption from the alimentary tract.



In general, sheep and poultry are relatively free from toxic effects, cattle are fairly resistant, while horses and pigs are more susceptible. In horses both haemolytic anaemia (Swales, Collier and Allen, 1942) and pathological changes in the urinary system (Swales, 1942) have been described after phenothiazine dosage. Certain individuals amongst man, dogs and cattle have also developed severe haemolysis after phenothiazine. Phenothiazine occasionally proved so toxic in man that its use has been discontinued. (Hubble, 1941). Sheep seem to be resistant to the haemolytic effects of the drug. Behrens (1950) described mild haemolysis in sheep after large doses of phenothiazine, but there were no clinical symptoms. Phenothiazine is not a particularly effective anthelmintic for pigs, and is seldom given to them now. It was usually given to these animals in food, where it was easy for greedy individuals to obtain an overdose. Cases of muscular inco-ordination, paralysis, and photosensitisation have been described in a small percentage of treated pigs. (Britton, 1943; Thorning, Morril and Boley, 1942).

Photosensitisation, mainly in the form of a keratitis, may also occur in cattle (Whitten and Filmer, 1947) if they are exposed to strong sunlight soon after dosage. Clare (1947) found that dosing calves with phenothiazine resulted in /



in the appearance of phenothiazine-sulphoxide, an oxidation product, in the aqueous humour, and Clare, Whitten and Filmer (1947) showed that this was the active agent in the photo-sensitised keratitis. Phenothiazine-sulphoxide did not normally appear in the aqueous humour of other species after phenothiazine dosage. Phenothiazine medication in dairy cattle is largely confined to calves, because coloured oxidation products of the drug are excreted in milk. Coloured oxidation products are also excreted in the urine of treated animals which means that in sheep there is a risk that the wool is stained.

Reports have been made of abortion in sheep after the administration of phenothiazine in late pregnancy (Warwick, Turk and Berry, 1946) but if it is given more than a month before lambing it is quite safe. (Gordon, 1945).

In the large scale manufacture of phenothiazine from sulphur and diphenylamine, iodine is used as catalyst because it speeds up the reaction and increases the yield. Variable amounts of readily available iodide ions are found in commercial preparations of phenothiazine as a resulting impurity. (Allcroft, Salt and Hignett, 1955). Reports that phenothiazine dosage reduced the uptake of radio-active iodine by the thyroid gland stimulated interest in the possibility /

possibility that phenothiazine had some thyroid-inhibiting activity. But Talmage, Monroe and Comar (1954) in sheep and Trum and Wasserman (1956) in horses showed that purified phenothiazine did not inhibit the uptake of iodine-131 by the thyroid. Talmage, Benson, Szafir, Turner and Allen (1956) concluded that the iodine content of commercial preparations was entirely responsible for the effect by diluting the radio-active material. Doses of iodine equal to the amounts found as impurities in phenothiazine had the same effect. (Trum and Wasserman, 1956). Pipes and Turner (1956) showed that prolonged dosage with commercial phenothiazine in cattle was not accompanied by a change in thyroid size or a decrease in output of thyroid hormone. Nevertheless Wasserman, Trum, Monroe, Lane and Comar (1956) obtained very slight inhibition of the thyroid uptake of iodine-131 with pure phenothiazine in chicks and rats and concluded that another factor was responsible for this slight effect.

It has been suggested tentatively that some of the weight gains obtained in phenothiazine-treated animals may be brought about by a reduction in thyroid function in cases of hyperthyroidism. (Talmage, Benson, Szafir, Turner and Allen, 1956).

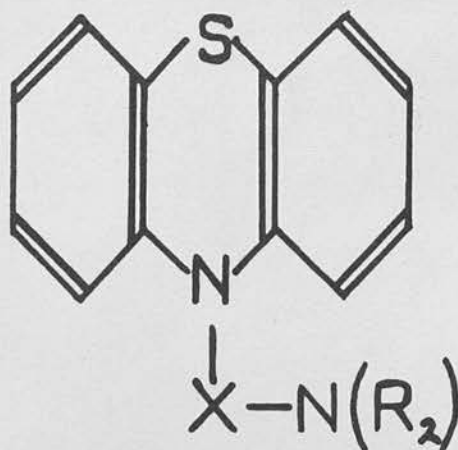
Phenothiazine /

Phenothiazine is unique amongst anthelmintics in that it reduces pasture contamination by preventing the development of infective larvae from worm eggs shed in the faeces of infected hosts. It also inhibits egg-laying by the female worms when given in doses too small to expel the parasites. (Shorb and Habermann, 1940). For this reason in some countries, small quantities of phenothiazine are supplied constantly either in salt-licks or mixed in the food. Foster and Habermann (1943 and 1944) showed that the drug provided in salt-licks gave effective control of infections in sheep and that small, frequent doses reduced infections in horses. Todd confirmed this finding in horses (1952) and showed that there were no pathological changes after horses had received 4 g/day for months. (1955).

In cattle, early results with low-level phenothiazine in the food were sometimes inconsistent because consumption of the mixture was uncertain. Mayhew (1950, 1951 and 1952) was able to show that when the intake of the drug was guaranteed the degree of infection was successfully controlled.

When the low-level administration of phenothiazine is carried out, it is supplemented at intervals with full therapeutic doses.

A number of phenothiazine derivatives are now used in therapeutics for their antiparkinsonism, local anaesthetic, or antihistaminic properties. These have the general formula



$X =$  divalent hydrocarbon chain

$-N(R_2) =$  aliphatic amine

None of these compounds have anthelmintic properties although most have diverse actions. (Viaud, 1954). Amongst the most important of these compounds are promethazine (an antihistamine) and chlorpromazine (an autonomic blocking agent and "narcobiotic"). Both these compounds /



compounds also have local anaesthetic properties. These amine derivatives of phenothiazine show fairly general anti-enzyme activity and they preserve tissues and blood. Decourt (1953) adopted the term "narcobiotic" to describe the diminution of cellular activity brought on by these drugs. He found the "narcobiotic" activity to apply to practically all living things from fungi and bacteria to mammals, and suggested that an inhibitory action on a dehydrogenase was responsible. What part the phenothiazine nucleus has to play in the activities of these compounds is unknown.

### Chemistry

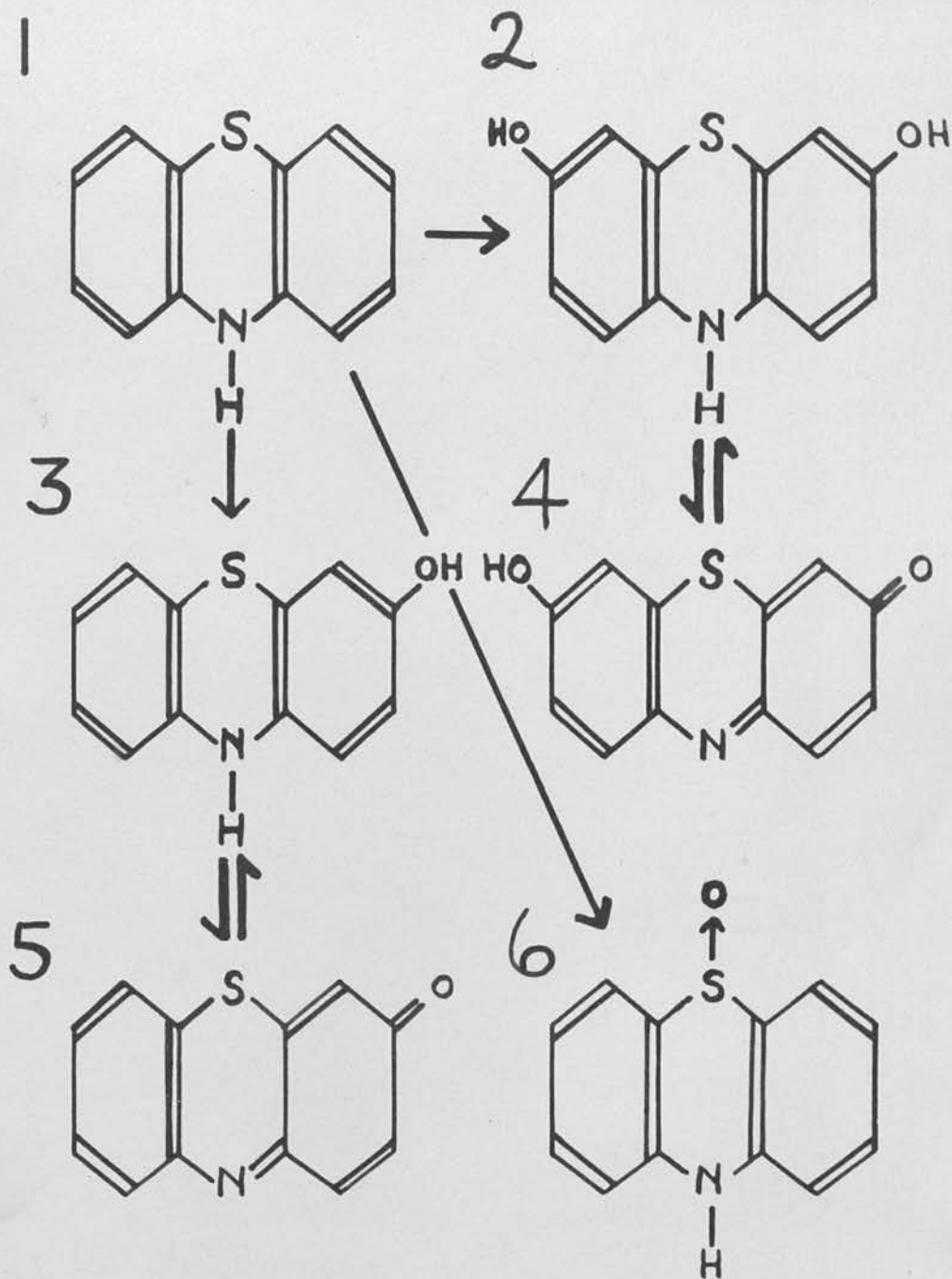
The chemistry of phenothiazine has been reviewed by Massie (1954). Pure phenothiazine is a greyish-yellow powder with a melting point which has been reported as 181-182°C. (Davey and Innes, 1942), 186-186.5°C. (Britton and Eisemann, 1942), and more recently as 185°C. (Harpur, Swales and Denstedt, 1950). It has a faint but bitter taste, has only a faint smell, and is almost insoluble in water; Davey and Innes (1942) found the solubility to be 1 in 800,000 parts water, and this has been confirmed by Harpur, Swales and Denstedt (1950). It is wetted only with difficulty, but is fairly readily soluble in a number of organic solvents. Commercial phenothiazine is a dark green powder containing dispersing agents and traces of oxidation products. Although phenothiazine is stable if kept free from light and moisture, it can be oxidised under appropriate conditions to a number of different compounds. Some of these are of especial interest because they are found in the body after the administration of phenothiazine. In an alkaline medium, phenothiazine is oxidised to phenothiazine-sulphoxide, a colourless compound, M.Pt. 250°C. (Harpur, Swales and Denstedt, 1950). In mild oxidising conditions, e.g. on exposure to light, air and /

and moisture, and in neutral or slightly alkaline conditions, phenothiazine is oxidised to the colourless leucophenothiazone, M.Pt.  $179-183^{\circ}\text{C}$ . (Harpur, Swales and Denstedt, 1950). This compound is the reduced form of the oxidation-reduction system phenothiazone-leucophenothiazone. The melting point of phenothiazone has been reported as  $161^{\circ}\text{C}$ . (Harpur, Swales and Denstedt, 1950).

In an acid medium and in stronger oxidising conditions phenothiazine is oxidised to leucothionol, the reduced form of the system thionol-leucothionol. Thionol has no melting point, and the melting point of leucothionol cannot be obtained by normal means because it is so readily oxidised to thionol, especially when heated.

Conversion of the colourless leuco compounds, leucophenothiazone and leucothionol, to the corresponding red dye forms, phenothiazone and thionol, occurs spontaneously if the oxygen pressure is above a certain limit; this process is accelerated in the presence of alkalis. When the oxygen pressure falls, reduction to the leuco compounds takes place.

The formulae of these compounds and their interactions are given on the following page.



- |                        |                                |
|------------------------|--------------------------------|
| 1. Phenothiazine.      | 2. Leucothionol.               |
| 3. Leucophenothiazone. | 4. Thionol.                    |
| 5. Phenothiazone.      | 6. Phenothiazine - sulphoxide. |



### Anthelmintic action

In spite of much work on the subject, the mode of action of phenothiazine on nematode parasites is completely unknown. Parasites expelled by the host after treatment with phenothiazine are not, as a rule, dead. They do not lose much of the compound when incubated in a phenothiazine-free medium after expulsion by the drug, and they live just as long in vitro as nematodes which have had no contact with the drug. (Lazarus and Rogers, 1950, 1951). It appears probable that the drug acts by temporarily paralysing or narcotising the worm so that it is expelled by the normal peristaltic movements of the host animal. One of the inexplicable properties of the compound is that to be effective in ridding an animal of parasites, it must be administered in extremely large doses. Because of this, and because phenothiazine apparently has little or no effect on nematode parasites in vitro, it appeared possible that one or more of its oxidation products was responsible for the anthelmintic effect. De Eds and Thomas (1941) claimed that although phenothiazine itself had no effect, thionol reduced the activity of Ascaris lumbricoides in vitro. On the other hand, Collier (1940)<sup>a</sup> found that neither phenothiazine nor thionol had any effect on Ascaris lumbricoides in vitro.

There /

There is little doubt that oxidation to leuco-phenothiazone and phenothiazine-sulphoxide takes place in the body, and some workers claim to have found leucothionol in the urine. (Lipson, 1940, De Eds and Thomas, 1942). There is less agreement about the presence of these derivatives in the alimentary tract. (Ellison and Todd, 1957; Harpur, Swales and Denstedt, 1950; Collier, Allen and Swales, 1943; Clare, 1947). A detailed account of the results of these workers will be given in a later Section.

It is probable that the active agent exerts its effect by inhibiting an enzyme system essential to the normal activity of the nematode. The oxidised derivatives of phenothiazine have been found to inhibit the activity of a greater number of enzymes than the parent compound. Leuco-phenothiazone, thionol and leucothionol have been shown to inhibit mammalian catalase and cytochrome oxidase. (Collier, 1940a). Phenothiazone has been shown to inhibit the activity of horse serum cholinesterase, (Collier and Allen, 1942a), and rat-brain hexokinase, (Allenby and Collier, 1952b). Both phenothiazone and thionol inhibit beef heart "succinoxidase" (Collier and Allen, 1942b) and phenothiazine-sulphoxide inhibits catalase (Collier and Allen, 1941). However, phenothiazine itself has some enzyme-inhibiting activity. Collier and Allenby /

• The enzyme now identified as succinic dehydrogenase, (Baldwin, 1957).

Allenby (1952) demonstrated that it was more effective than phenothiazine sulphoxide or phenothiazone in inhibiting "succinoxidase" activity of rat liver mitochondria, while thionol had only a slight inhibitory effect on this enzyme. Collier (1953) showed that phenothiazine as well as phenothiazone inhibited the glyoxalase activity of human and rabbit red blood corpuscles.

These properties may help to account for some of the after-effects of phenothiazine dosage. Inhibition of glyoxalase activity by phenothiazine and phenothiazone would interfere with the normal metabolism of erythrocytes and might be responsible for the haemolytic anaemia sometimes observed after phenothiazine dosage. (Collier, 1953). However, a more probable explanation for the haemolysis has been forwarded by Collier and Allen (1942c) who found that phenothiazone and especially potassium leucophenothiazone sulphate accelerated the haemolytic effect of certain lysins on horse erythrocytes in vitro. These lysins are normally found in the blood. Sheep erythrocytes were relatively resistant to haemolysis by these agents. The inhibition of cholinesterase activity by phenothiazone may account for the nervous symptoms seen in overdoses or susceptible pigs after phenothiazine dosage. (Collier and Allen, 1942a).

Possible /

Possible mechanisms for toxic actions on helminths are more difficult to suggest, especially as much less is known about their metabolism, but the inhibition of hexokinase by phenothiazone could be of significance, as the majority of helminths, being anaerobic, may obtain their energy through glycolysis. Most of this work on enzymes has been carried out on those of mammals; unless similar results are found to apply to nematode enzymes, any theory as to the exact mechanism of action of phenothiazine must be without a solid foundation.

In spite of their greater activity on enzymes it appears unlikely that the known oxidation products of phenothiazine are the actual anthelmintics. Phenothiazone (Collier, Allen and Swales, 1943; Taylor and Sanderson, 1940), and thionol (Gordon and Lipson, 1940; Taylor and Sanderson, 1940) have been administered to parasitised animals without anthelmintic effect. Phenothiazine-sulphoxide has anthelmintic properties (Whitten, 1948, 1956) but it is unlikely to be the active agent as it must be given in similar doses to phenothiazine to be effective. Moreover, only Clare (1947) has found phenothiazine-sulphoxide in the alimentary tract after phenothiazine dosage. Gordon (1948) noted the interesting fact that Oesophagostomum columbianum were expelled more rapidly from sheep after phenothiazine-sulphoxide than after phenothiazine. If phenothiazine itself /



itself is not the active compound the possibility remains that part of the dose is converted in the body to an as yet unidentified compound. Harwood (1953) has suggested that an unoxidised derivative may be responsible for the anthelmintic effect.

If the whole of a dose of phenothiazine could be accounted for as phenothiazine and its known oxidation products this theory would have to be discounted.

To determine the fate of phenothiazine in the body, various workers have carried out quantitative determinations of phenothiazine and its oxidation products in the tissues and excreta of dosed animals. Quantitatively their results have been variable. (Swales and Collier, 1940; Lipson and Gordon, 1940; Harpur, Swales and Denstedt, 1950; Collier, Allen and Swales, 1943).

Initially, an attempt was made to employ some of the methods other workers had used. However, none of these were found to be satisfactory. New methods were therefore devised and quantitative estimations carried out on the excreta of phenothiazine-dosed sheep and rabbits. Up to the present little attention seems to have been paid to the fate of the known oxidation products. Therefore sheep were dosed with each of these derivatives, and their fate in the body followed. In /

In order to help establish whether phenothiazine is oxidised in the alimentary tract it was decided to incubate phenothiazine with sheep rumen contents and examine extracts for the presence of oxidation products. Phenothiazine was also incubated with horse dorsal colon contents.

When the work with phenothiazine was begun it was necessary to find a method which could be used to separate phenothiazine and its oxidation products.

SECTION II

PART I

Preliminary Investigation of Methods of  
Separation of Phenothiazine and its Derivatives

Methods

In order to find a suitable method for separation of the compounds it was necessary to know their relative solubilities in various common solvents. Some information about these properties was obtained in the literature. (Beilstein, 1913+). This is presented in tabular form (page 36). As the data obtained was incomplete, the relative solubility of phenothiazine, phenothiazone, thionol and phenothiazine-sulphoxide in fourteen common solvents was determined and a table of results is given on pages 37 and 38. The colour of each solution is also given. These tables were found to be invaluable when different methods of separation were investigated. A method which appeared to hold out promise for separation of the compounds was that of chromatography. Lipson (1940) used alumina columns for the separation of phenothiazone and thionol in sheep urine. In these experiments, however, satisfactory recoveries were not obtained when this method was employed.

TABLE I

Solubility of Phenothiazine and some of its Derivatives in a Variety of Solvents

(Data obtained from Beilstein, 1913+)

	Phenothiazine	Thionol	Leuco-thionol	Phenothiazone	Leuco-phenothiazone	Phenothiazine-sulphoxide
	Mpt. 180°C			Mpt. 165-6°C	Mpt. 172-3°C	Mpt. (approx.) 250°C
<u>Solubility in</u>						
Water	v. difficult	v. difficult	v. difficult	xxx (boiling)		
Dilute acids		xxx	xxx	xxx		
Dilute alkalis		xxx	xxx	insoluble		
Conc. acids	H <sub>2</sub> SO <sub>4</sub>	HCl & H <sub>2</sub> SO <sub>4</sub>		H <sub>2</sub> SO <sub>4</sub>	xxx	H <sub>2</sub> SO <sub>4</sub>
Ethyl alcohol	x (cold)	xxx	xxx	xxxx		
Chloroform		v. difficult				
Ether	xxx				xxx	
Benzene	xxxx	v. difficult		xxx	xxx	
Acetone						
Toluene						
Pet. ether	v. difficult	v. difficult				
Aniline						
Melted Naphthalene						
Acetic acid (glacial)	xxx (hot)	xx	xx	xxx	xxx	
Ammonia		xxx	xxx			
Sodium acetate		xxx	xxx			

xxxx Easily soluble

xxx Soluble, or fairly readily soluble

xx Slightly soluble

x Soluble with difficulty



TABLE II

Relative Solubility of Phenothiazine and some of its Derivatives, giving Colours in Solution  
(Personal Observations)

	Phenothiazine		Thionol		Phenothiazone		Phenothiazine-sulphoxide.	
	Solubility	Colour in solution.	Solubility	Colour in solution	Solubility	Colour in solution	Solubility	Colour in solution
Water	$\frac{+}{-}$ insoluble	-	v. difficult	v. faintly blue	x (cold) xxx (boiling)	purplish-pink	$\frac{+}{-}$ insoluble (hot and cold)	-
Dilute acids	x	pinkish	xx	purple-red	xx	red	xx	red-brown
Dilute alkalis	$\frac{+}{-}$ insoluble	-	xxx (not in strong alkali)	intense purple-blue	x	pinkish	insoluble	-
Conc. acids	xxxx	H <sub>2</sub> SO <sub>4</sub> dark green-red-brown	xxx	Intense purple-blue	H <sub>2</sub> SO <sub>4</sub> xxxx	red-green	xxxx	green
Ethyl alcohol	xxx	v. pale yellow-colourless	xxx	reddish	xxxx	red	xx cold xxx hot	-
Chloroform	xxxx	pinkish	xx	red	xxxx	red	xx cold	pink-
ether	xxx	colourless	xx	yellow-pink	xxxx	orange-red	$\frac{+}{-}$ insoluble (hot or cold)	-
Benzene	xxxx	v. pale yellow-colourless	v. difficult	v. faintly pink	xxxx	red	$\frac{+}{-}$ insoluble (hot or cold)	-
Acetone	xxxx	v. pale yellow-colourless	xx	red	xxxx	red	xx	colourless
Toluene	xxx	yellow	x	yellowy-pink	xxxx	red	$\frac{+}{-}$ insoluble (hot or cold)	-
Pet. ether	x (cold) xx (hot)	colourless	$\frac{+}{-}$ insoluble	-	xx	orange	insoluble (hot or cold)	-
Xylol	xxxx	v. pale yellow-colourless	x	yellowy-pink	xxxx	red	insoluble (hot or cold)	-

TABLE II (Contd.)

Relative Solubility of Phenothiazine and some of its Derivatives, giving Colours in Solution

	Phenothiazine		Thionol		Phenothiazone		Phenothiazine-sulphoxide	
	Solubility	Colour in solution	Solubility	Colour in solution	Solubility	Colour in solution	Solubility	Colour in solution
Ethyl acetate	xxx	v. pale yellow - colourless	xxx	yellowy-pink	xxx	red	insoluble (hot or cold)	-
Carbon tetrachloride	xx (hot)	yellowy-pink	v. difficult		xxx	orange-red	insoluble (hot or cold)	-

xxxx Easily soluble.  
 xxx Fairly readily soluble.  
 xx Slightly soluble.  
 x Soluble with difficulty.

### Paper chromatography

A method suggested by Williams (1954) using filter paper circles was adopted for screening a number of solutions for their ability to separate the compounds on paper. The method was simple to set up and results were seen quickly. It had the additional advantage that several tests could be carried out simultaneously.

#### Materials and Methods.

Whatman No. 1 filter paper circles, 12.5 cm. in diameter were used. For each test two glass plates about 14 cm. square were required, one of which had a hole 0.4 mm. in diameter in the centre.

A small drop of a concentrated solution of the compound or compounds under investigation was applied to the centre of a filter paper disc and allowed to dry. Care was taken not to overload the paper. Two parallel cuts 0.2 - 0.3 mm. apart were then made in the paper, extending from the circumference to the centre of the circle. The resulting strip was cut off about 2.5 cm. from the centre. The filter paper was then put between two glass plates so that the shortened strip projected through the hole in one of them.

The system was then placed over a petri dish containing a watch-glass full of the solution under examination /



examination so that the filter paper tongue acted as a wick, drawing up the solution. (See illustration, Page 42). When the solvent front was 1-2 cm. from the circumference of the circle, the paper was removed, dried and examined. The length of time taken for the formation of the chromatogram varied with the nature of the solution used; as a rule it was not less than three quarters of an hour and not more than two hours. When separation was carried out with a watery solution more satisfactory results were obtained if a weight was placed on the upper glass plate, otherwise the paper tended to develop waves. This was unnecessary when organic solvents were used. Because phenothiazine oxidises easily in moisture, light and air, development was carried out in the dark. The system was examined at intervals, and when development was complete the paper disc was removed, dried and examined. If the substances were separated, concentric circular zones could either be seen or be shown up with appropriate reagents. Identification of the substances to be separated on the paper could be established by their relative distances from the solvent front, by the colour of the zones, or by the colour developed by the zones under certain conditions. Pure samples of phenothiazine, phenothiazone, thionol and phenothiazine-sulphoxide were dissolved in suitable solvents, and applied to the papers both separately and together. A number of solutions were used to develop the papers to find out which would separate the four compounds.

The /

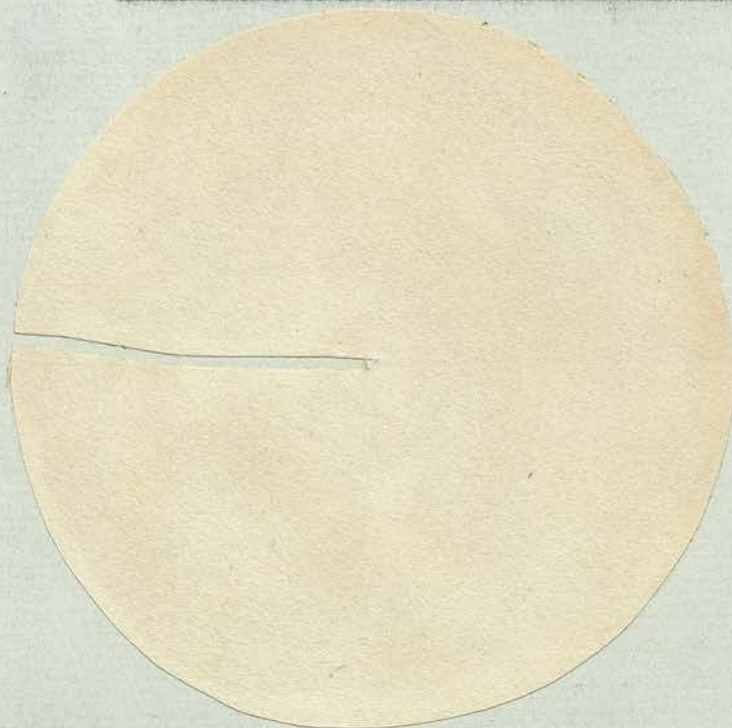


The position on the paper, colours or colour reactions of each compound after development with various solvents was noted. Altogether more than thirty solvents or mixtures of solvents were tried out for their developing properties. A list of these is given on page 42a. When a satisfactory developing fluid for the separation of phenothiazine, phenothiazone, and thionol was found by this method, it was hoped that the same fluid would separate the compounds when using large sheets of paper in tanks. This would have allowed larger quantities of the substances to be separated, so that elution and quantitative estimation could be carried out.

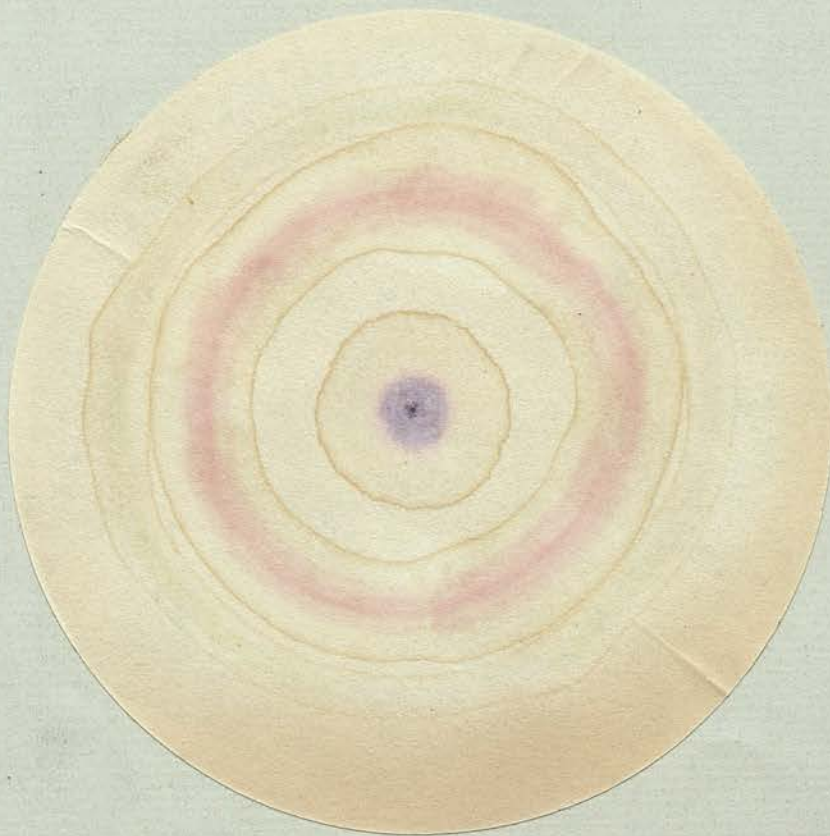
Satisfactory separation was not obtained on a larger scale, however, the zones being too diffuse even when the fluids were modified, the type of paper was altered, and the development made slower by various means. Separation was also attempted by the use of glass columns tightly packed with cellulose powder, the compounds to be separated being applied in a few drops of a concentrated solution and the developing solution then being poured into the column. Using this method, percolation and elution were very slow, and separation unsatisfactory. It was decided that development with a fluid using filter paper circles would provide a useful test to identify the contents of extracts of biological material.

If an extract appeared to contain a single phenothiazine compound, identification was confirmed by the use of "co-chromatography". (Stepka, 1952). A little of the appropriate pure compound was dissolved /

Actual paper chromatogram of Phenothiazone and Thionol after development with Petroleum ether and Acetone.

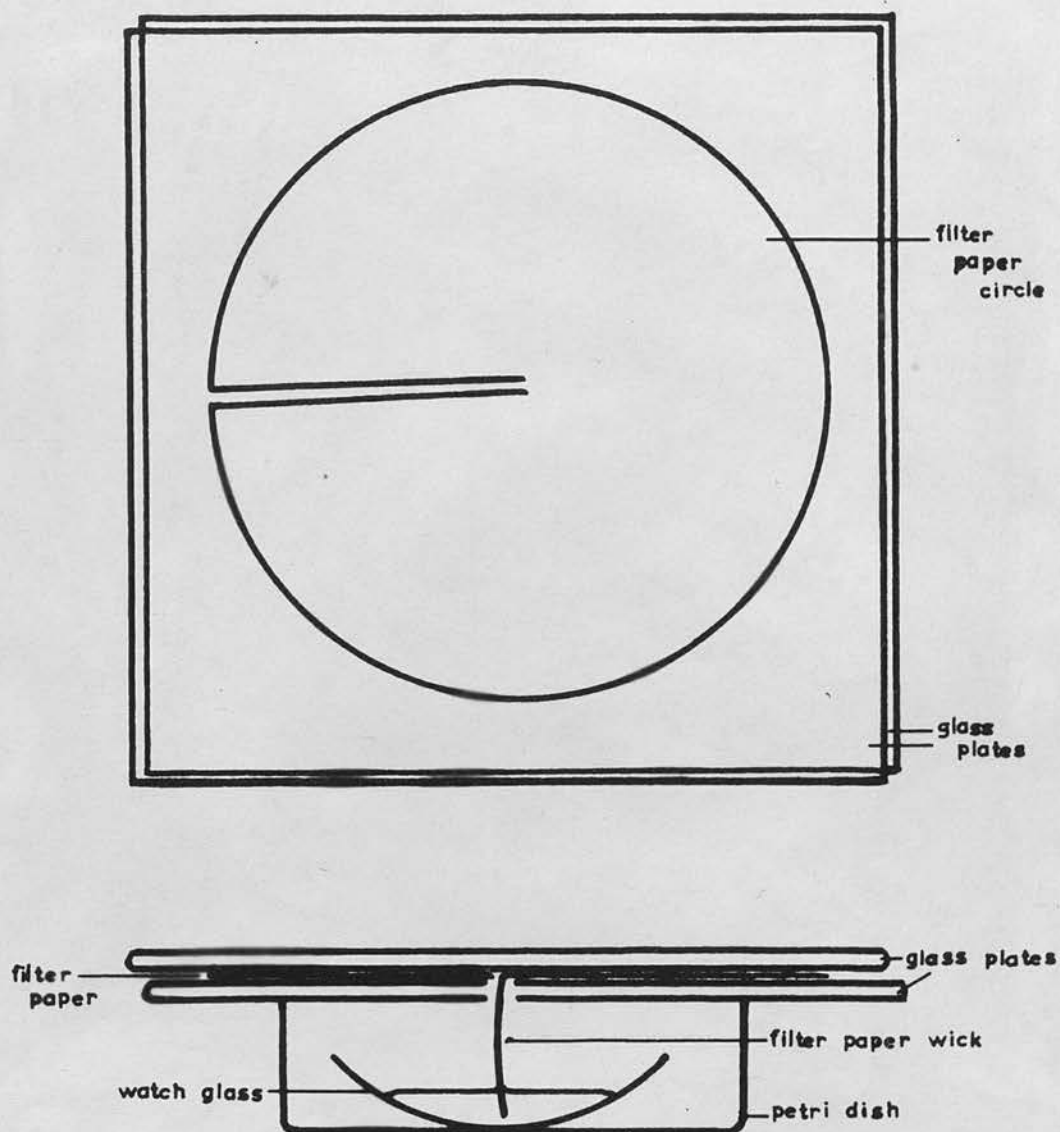


Painted reproduction of original colours.



dissolved in the extract and the mixture developed with dissimilar solvents on several papers. If the two substances always ran together, producing a single zone, then the two compounds were assumed to be identical.

Apparatus for Paper Chromatography.





Solvents or Mixtures of Solvents used to try to separate phenothiazine, phenothiazone, thionol and phenothiazine-sulphoxide by paper chromatography.

- |  |  |
|--|--|
| 1) Water                                       | 19) Ether (saturated with water)           |
| 2) Acetone                                     | 20) Petroleum ether (saturated with water) |
| 3) Ethyl alcohol                               | 21) Water saturated with petroleum ether   |
| 4) Chloroform                                  | 22) Propylene glycol and water             |
| 5) Diethyl ether                               | 23) n-butyl alcohol, acetic acid and water |
| 6) Benzene                                     | 24) Toluene saturated with water           |
| 7) Xylol                                       | 25) Toluene and ethyl alcohol              |
| 8) Toluene                                     | 26) Toluene and acetone                    |
| 9) Petroleum ether                             | 27) Xylol and acetone 1:1                  |
| 10) n-butyl alcohol<br>(saturated with water)  | 28) Xylol and acetone 2:1                  |
| 11) n-propyl alcohol<br>(saturated with water) | 29) Xylol and acetone 1:2                  |
| 12) Propylene glycol                           | 30) Petroleum ether and acetone 1:2        |
| 13) N/50 Hydrochloric acid                     | 31) Petroleum ether and acetone 1:1        |
| 14) Dilute Sulphuric acid                      | 32) Petroleum ether and acetone 2:1        |
| 15) Dilute Sodium hydroxide                    | 33) Petroleum ether and acetone 4:1.5      |
| 16) Glacial acetic acid                        | 34) Petroleum ether and acetone 6:1        |
| 17) Dilute acetic acid                         | 35) Petroleum ether and acetone 10:1       |
| 18) Chloroform (saturated<br>with water)       | 36) Petroleum ether and acetone 8:1        |



### Results

When phenothiazone was applied to paper in solution and dried, it appeared as a pink zone. The pink colour faded slowly when the paper was kept exposed to light but could be preserved in the dark. It ran at the solvent front when developed with acetone and chloroform, further back with alcohol and toluene. The zone formed was rather diffuse after development with alcohol.

A thionol zone was a faint bluish colour which became intensely blue when the paper was exposed to ammonia vapour. The blue colour faded fairly rapidly but could be restored with ammonia vapour. Thionol generally moved very little on development with organic fluids. It was easily separated from phenothiazone in acetone, alcohol and chloroform.

A zone containing phenothiazine was colourless when first formed but slowly turned green on exposure to light and air. The application of concentrated sulphuric acid to the paper produced a brownish-pink colour immediately if phenothiazine was present. Phenothiazine tended to run towards the solvent front with organic developing fluids. It was easily separated from thionol in alcohol and petroleum ether, but only petroleum ether, of the fluids tested, would separate it from phenothiazone.

Phenothiazine /

Phenothiazine-sulphoxide again showed no colour on the paper, but when concentrated sulphuric acid was applied, the momentary appearance of a bright greenish colour revealed its presence. It appeared in the same zone as phenothiazine after development with alcohol, but in the same zone as thionol after development with petroleum ether or acetone.

Many solvents or mixtures of solvents were unsatisfactory as developing agents because they spread the compounds over the paper instead of forming distinct zones. The most satisfactory fluid found to separate phenothiazine, phenothiazone and thionol was a mixture of eight parts petroleum ether to one part of acetone. No solution was found which would separate all four compounds. Using this developing fluid, the phenothiazine appeared near the solvent front, the pink phenothiazone half-way to the solvent front, and the blue thionol remained at the spot of application. Provided the paper was not overloaded with materials to be separated the separation was very satisfactory.

Sometimes the normal coloured constituents of an extract of faeces or intestinal contents appeared as interfering zones but these faded when the paper was left exposed to light and air. The method was simple to set up and /

and results were seen quickly. Several tests could be carried out simultaneously, and only small amounts of material were required. Unfortunately the quantities dealt with were too small to allow elution and quantitative estimations to be made.

It was possible to store the paper discs, suitably labelled, for reference. If kept in the dark, the zone of phenothiazone retained its colour, while those of thionol and of phenothiazine could be restored by ammonia vapour and exposure to daylight respectively.

SECTION II

PART II

Main Investigation of Phenothiazine and its Derivatives

Methods

Metabolic      The sheep and rabbits used in these experiments were kept in metabolism cages which have been described in detail by Hood (1957). These were made so that the urine and faeces fell through a mesh floor and ran down a steep incline until a finer mesh was reached. The urine passed through this completely into a receiving vessel, while the faecal pellets were deflected into a separate container. This arrangement separated urine and faeces very satisfactorily if the following precautions were taken.

The animals were kept in the cages for several days before they were dosed to accustom them to their new surroundings and diet. Failure to do this often resulted in the appearance of soft faeces which adhered to the mesh on the floor of the cage.

Water was provided in unspillable containers; the rabbits were fed on bran and oats and the sheep crushed oats and chopped hay. If long hay was given it was soon scattered and interfered with the separation of urine and faeces.

Phenothiazine /



Phenothiazine or one of its known oxidation compounds was dissolved in propylene glycol, then suspended in water and administered by stomach tube. Rubber tubing (outside diameter 12 mm., inside diameter 6 mm.) was used for the sheep stomach tube; for the rabbits a No. 9 soft rubber catheter was found to be suitable. In both cases the tube was lubricated with a few drops of glycerine.

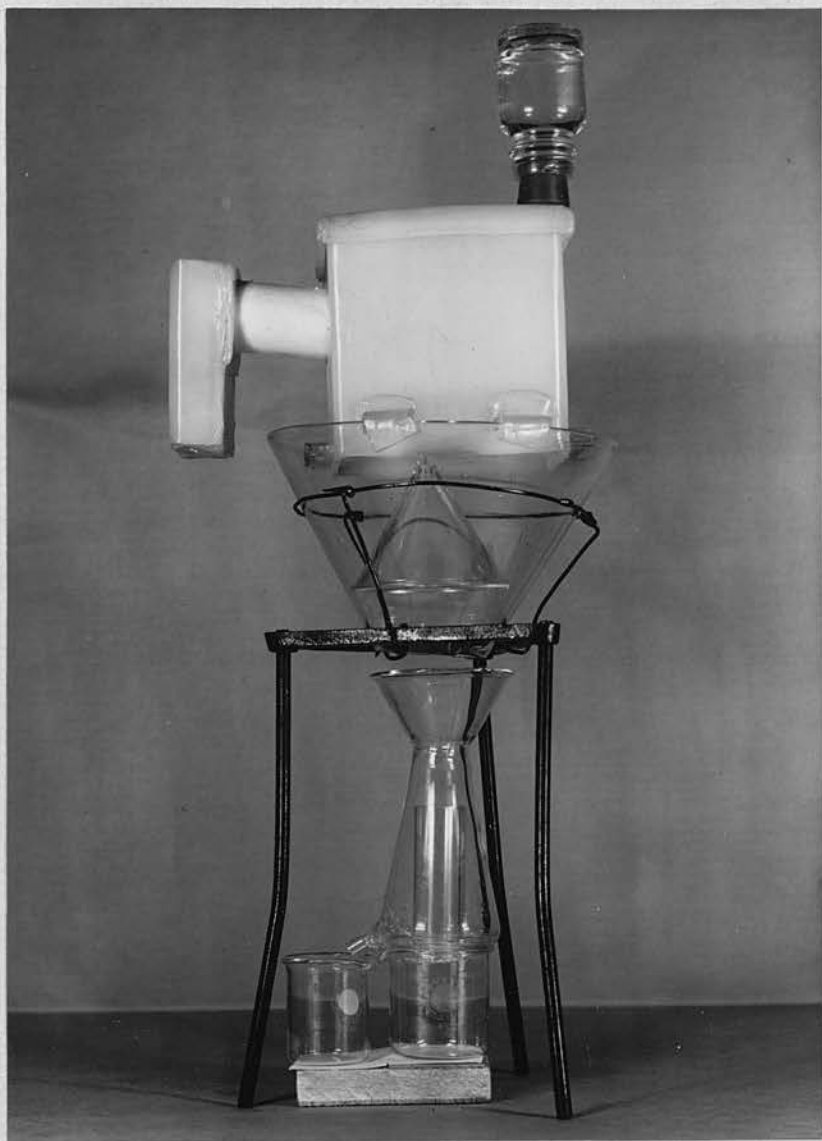
After receiving the appropriate dose the animals were kept in their respective metabolism cages until the urine and faeces were without trace of phenothiazine compounds, usually five or six days after dosing. During this time the urine and faeces were collected, measured and analysed for phenothiazine and its known oxidation products. Faeces and urine from undosed sheep and rabbits, as well as from those given propylene glycol, were also examined. The sheep were half-bred wethers aged from ten to fourteen months, weighing between 59 and 65 kg.

Mice were kept in a metabolism cage made of sheet polythene, the floor consisting of parallel lengths of glass rod 0.5 cm. in diameter, placed 1 cm. apart. A water bottle was provided for the mice and a container for food placed so that the mice had to crawl along a small tunnel to eat. Because this did not stop them from spilling dry food into the /

the separator, the mouse nuts were ground up into a powder and given after incorporation into a jelly. The separator is a modification of that described for rats by Lazarow (1954). The complete assembly is illustrated on page 49. Various types of separator were tried out; the one illustrated was much the most satisfactory. The urine was deflected by the small inverted funnel on to the walls of the large one, then ran down the glass sides of the system into a beaker. The faeces dropped down the centre hole into another container. The mice were dosed with from 300 - 3000 mg/kg. phenothiazine. To some it was given by stomach tube dissolved in propylene glycol. The stomach tube was made from portex tubing, (outside diameter 1.5 mm. inside diameter 1 mm.). The urine and faeces were collected for forty-eight hours after the mice were dosed. The mice were then killed and their organs removed for analysis. Controls were made with undosed mice and others given propylene glycol alone.

Chemical /

Mouse Metabolism Cage



## Chemical

### Estimation of Phenothiazine

Phenothiazine was purified by the method of Kniasseff (1948). The product melted at 182°C. The particle size of the purified phenothiazine when it was precipitated from propylene glycol solution with water was estimated with the aid of an ocular micrometer. The average particle size appeared to be about 30  $\mu$ . There were many particles as small as 1  $\mu$  or smaller, but a few particles were as big as 150  $\mu$ . The particle sizes of a commercial preparation examined were even more variable. Great care was taken to prevent the accidental oxidation of phenothiazine before estimation. Solutions were kept in the dark as much as possible, and estimations carried out without delay.



a) In intestinal contents and faeces      The semi-solid biological material was ground up with anhydrous sodium sulphate, using a pestle and mortar, until a powdered product was obtained. This powder was then carefully transferred to a Soxhlet thimble and extracted with petroleum ether (boiling-point  $40^{\circ} - 60^{\circ}\text{C}$ ), heated in a water-bath kept at  $65^{\circ}\text{C}$ . Preliminary experiments had revealed that petroleum ether was more satisfactory than acetone, toluene and a number of other solvents for this purpose. Extractions were carried out for varying times until the optimum was found. When the extraction was complete the petroleum ether was evaporated off in vacuo and the residue extracted with hot 95% ethyl alcohol. The resulting solution was cooled, filtered into a volumetric flask and diluted if necessary. It was then brominated at  $60^{\circ}\text{C}$  according to the method of Cupples (1942) and the intensity of the red colour produced was compared with that of a similarly treated standard solution by taking readings in a Unicam S.P. 350 Diffraction Grating Spectrophotometer. The wave-length of maximum sensitivity was found to be 505 millimicrons. When estimations were made on the faeces of dosed animals, the faeces were collected daily, weighed, thoroughly mixed and a weighed aliquot removed for analysis.

Control /



### Control Experiments

Control readings were necessary because the extracts contained variable amounts of coloured substances normally present in faeces (or digestive tract contents). Because bromination partly bleached the interfering substances, control readings were made on each extract before bromination. These readings were then corrected by reference to a graph which had been made from readings of normal faeces extracts before and after bromination. Attempts were made to remove the coloured faecal constituents from the extracts by shaking them with "Norit", a form of activated charcoal. This proved unsuccessful because the charcoal removed both the interfering substances and phenothiazine from the extracts.

b) In urine      A mixture of phenothiazine, phenothiazone and thionol dissolved in toluene or added to urine was subjected to steam distillation. The distillate and the liquid which remained behind were both retained for analysis. The distillate was acidified with hydrochloric acid and shaken twice with warm petroleum ether. The petroleum ether layer was evaporated to a small volume and a paper disc chromatogram made. Only phenothiazine was present. The petroleum ether was evaporated off, the residue dissolved in 95% alcohol, brominated and estimated colorimetrically. The /

The fluid which remained in the boiling flask when steam distillation was complete was purplish in colour. It was acidified with hydrochloric acid and shaken with chloroform. The chloroform extract on analysis by paper disc chromatography showed only phenothiazone and thionol.

Many workers, e.g. Lipson (1940) have shown that phenothiazine is excreted in urine largely in the form of an unidentified conjugate from which it can be released by acidification. Phenothiazone and thionol are known to be excreted in urine as their colourless leuco forms, although once the urine has been voided and is on contact with light and air, oxidation begins to take place at once. (De Eds and Thomas, 1942). To simplify matters, the derivatives extracted from urine will be referred to as phenothiazone and thionol.

Measured aliquots of urine from phenothiazine-dosed rabbits were acidified and steam distilled to separate phenothiazine from phenothiazone and thionol. No thionol was found in the rabbit urine when the dose of phenothiazine was fairly small. Phenothiazone was recovered after repeated extractions with chloroform, allowing the washings to be exposed to light and air for some hours between successive extractions to allow the leuco-phenothiazone to oxidise to phenothiazone. Bubbling air through the solution accelerated this process. The phenothiazone was identified by its ultra-violet /

violet and visible absorption spectra after bromination at 60°C in 95% alcohol. Absorption spectra were obtained on a Hilger Uvispek photoelectric spectrophotometer. The alcohol had to be refluxed with sodium hydroxide and distilled twice before it was pure enough to be used as a solvent for ultra-violet spectrophotometry. The maxima were shown to be identical with those for pure phenothiazine after bromination. (Harpur, Swales and Denstedt, 1950).

Separation, Purification and Estimation of Phenothiazine and Derivatives in Urine and Faeces after Administration of Phenothiazine Derivatives

a) Separation and Purification Because recoveries from steam distilled urine were low, other methods were adopted, using the solubility tables (pages 37 and 38) as a guide. The urine was evaporated slowly almost to dryness, stirred with warm alcohol and filtered. The filtrate and residue were separately extracted with hot chloroform and a few drops of the extracts subjected to paper chromatography. Chloroform was the solvent of choice because most of the solids in normal urine are insoluble in it.

Soxhlet extraction of the faeces of sheep which had been dosed with phenothiazine derivatives was carried out with hot petroleum ether which would be expected to extract any phenothiazine or phenothiazone present. The residue left in the Soxhlet /



Soxhlet thimble was re-extracted with hot water and then with very dilute sulphuric acid. The resulting liquid was further acidified to pH 1.0 with sulphuric acid and filtered. Hot chloroform extracts of the filtrate and residue were subjected to paper chromatography, as was the petroleum ether extract, to determine which, if any, phenothiazine derivatives were present. The presence of coloured impurities was revealed by the appearance of zones other than those expected from phenothiazine compounds. Steps were then taken to separate and purify any phenothiazine compounds present in the extracts. Any phenothiazine sulfoxide in the faeces was separated from thionol by shaking the acidified filtrate with chloroform (thus removing thionol) making the remaining filtrate alkaline with sodium hydroxide solution and again shaking with chloroform. The thionol appeared in the first chloroform extract and phenothiazine sulfoxide in the second.

Thionol was separated from other materials and thereby purified by making the extract alkaline with sodium hydroxide solution, filtering, acidifying the filtrate and shaking it with chloroform. This was repeated if necessary until paper chromatograms showed that the extract contained only thionol.

Phenothiazine and phenothiazone together were more difficult to separate although in alkaline solution chloroform extracted most of the phenothiazone present.

b) /

b) Identification and Estimation When pure phenothiazone was obtained in any extract, its melting-point, mixed melting-point, and absorption spectrum were determined. The quantity present could be estimated by comparing the reading at a given wavelength with that given by pure phenothiazone, or by weighing the dried extract.

Phenothiazine was identified by its melting-point, and the quantity was estimated by colorimetric means. Thionol was identified by its absorption spectrum, and an estimate of its concentration could also be obtained from this data. Alternatively, the solvent was evaporated off, the residue dried in vacuo over calcium chloride and the product weighed. Phenothiazine-sulphoxide was identified tentatively by the greenish colour developed initially when a trace was brought in contact with concentrated sulphuric acid, then by its melting-point.

Examination of rabbit liver, kidneys and bile

A rabbit was killed eighteen hours after receiving 266 mg/kg phenothiazine by stomach tube. The liver and kidneys were homogenised separately and extracted with hot petroleum ether and then with ethyl alcohol. The bile was collected and was shaken with chloroform. These extracts were then examined by paper chromatography.

Examination /

Examination of urine, faeces, and various organs of dosed mice

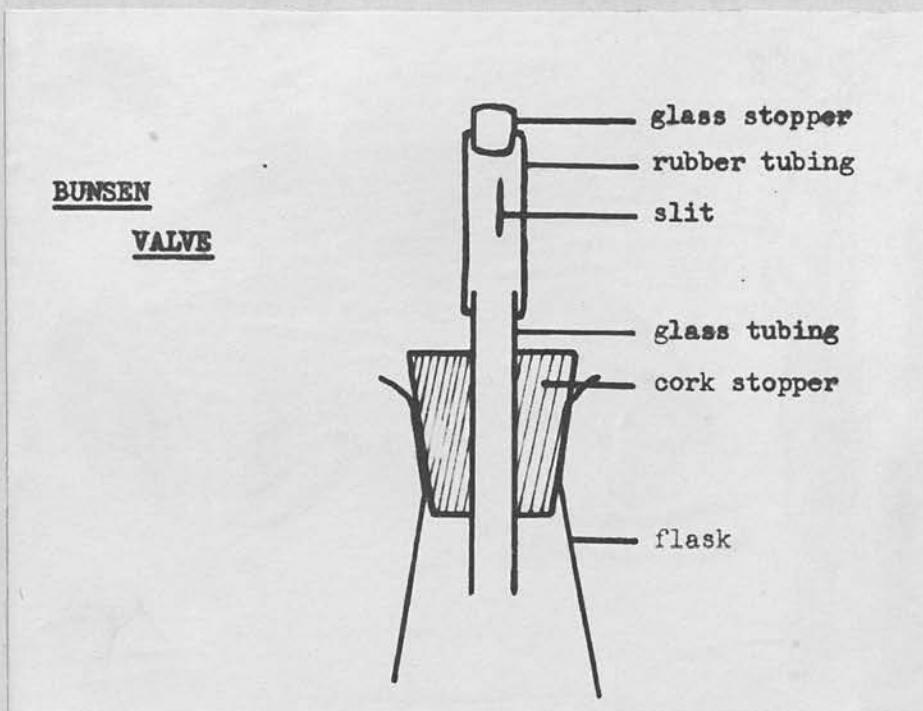
The urine and faeces from mice dosed with phenothiazine were dealt with in the same way as those from rabbits, i.e. the urine was steam distilled, and the faeces extracted with petroleum ether. When the mice were killed, forty-eight hours after the dose was given, the livers, kidneys, stomachs, intestines, and intestinal contents were homogenised separately and extracted with hot petroleum ether, ether and alcohol. The other parts of the mice were cut up into small pieces, either dried in an oven (60-80°C) or mixed with anhydrous sodium sulphate, then extracted with petroleum ether in a Soxhlet apparatus. All these extracts were then subjected to examination by paper chromatography. If phenothiazine was present alone in any of these extracts it was estimated colorimetrically. Controls were made with undosed mice.

Incubations

## Incubations

### Methods

The incubations were carried out in 100 or 50 ml. Ehrlemeyer flasks fitted with Bunsen valves. The slit in the rubber tubing will allow gases to escape but not to enter.



Horse dorsal colon contents or sheep rumen contents obtained from animals provided with fistulae were put in these flasks and incubated with phenothiazine at  $37^{\circ}\text{C}$  for eighteen hours. After incubation, the contents of the flasks were mixed with anhydrous sodium sulphate and extracted with petroleum ether in a Soxhlet apparatus for twenty-four hours.

Hot /



Hot water extracts were made of the residues. The extracts were concentrated and drops of each subjected to paper chromatography. Controls were made with boiled intestinal contents; without phenothiazine; with distilled water instead of intestinal contents; and with the contents of the flask kept at 4°C.

## Results

To determine the accuracy of the method for estimating phenothiazine, weighed amounts of pure phenothiazine were brominated and estimated colorimetrically in 95% alcohol.

Typical recoveries are shown in Table III (page 61) and the graph on the following page, showing that Beer's Law is followed, and that the method is accurate for pure solutions within  $\pm 5\%$ .

Evidence was obtained that the presence of phenothiazone or thionol in alcoholic solution interfered with the colorimetric estimation of phenothiazine. When alcoholic solutions containing phenothiazone, thionol and phenothiazine sulphoxide respectively were brominated, colour changes took place as follows:

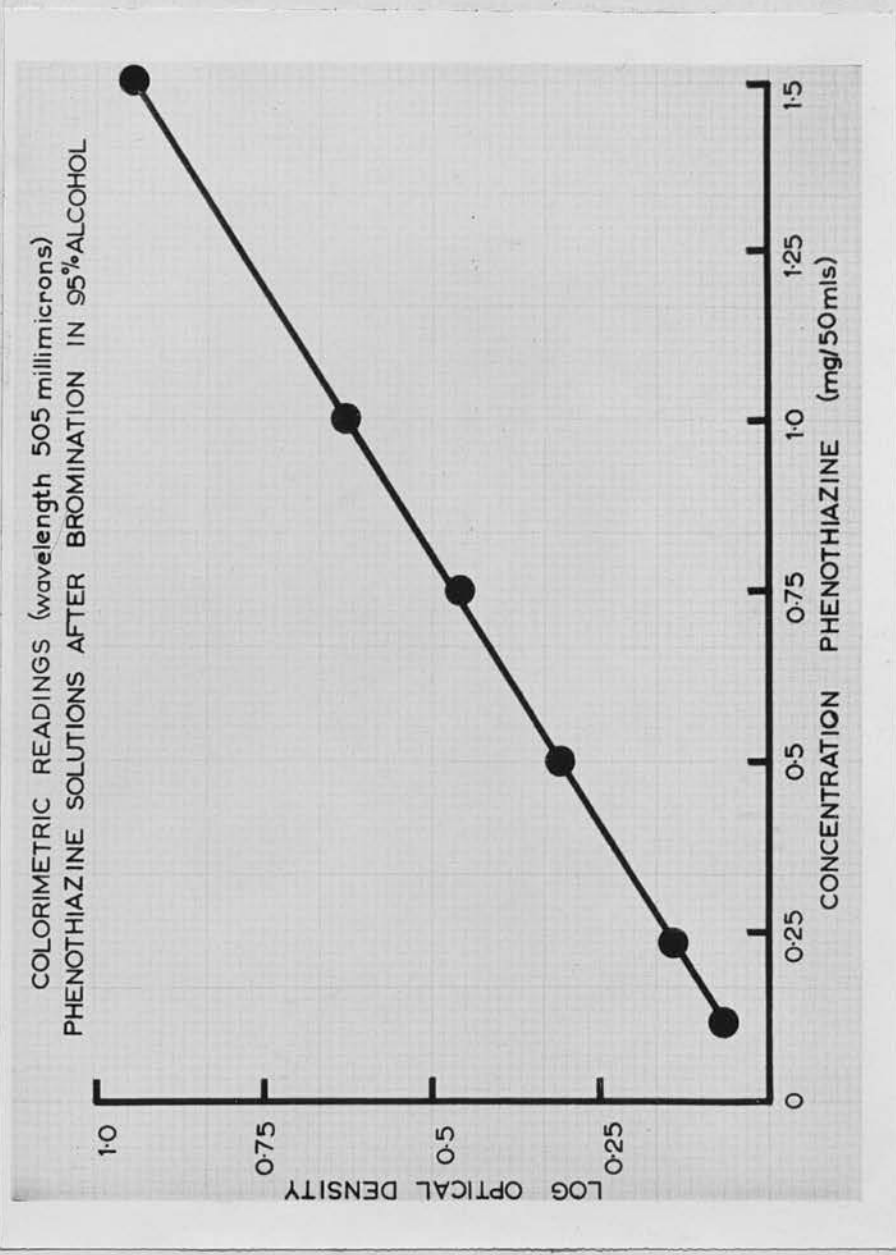
Phenothiazone	-	Bright red	-	very dark red
Thionol	-	Bright red	-	dark red
Phenothiazine				
sulphoxide	-	Colourless	-	pale pink

Four 50 ml. volumetric flasks containing 0.5 mg. phenothiazine in 25 ml. 95% alcohol were taken. To one flask was added 0.5 mg. phenothiazone, to another 0.5 mg. thionol, to another 0.5 mg. phenothiazine-sulphoxide. The fourth flask was kept as a control. The contents of the flasks were brominated, diluted to 50 ml. and read in the spectrophotometer at wavelength 505 m $\mu$ .

Results /

**TABLE III**  
**Colorimetric Readings of Pure Phenothiazine Solutions**  
**(Wavelength 505)**

CONCENTRATION OF PHENOTHIAZINE SOLUTION	COLORIMETRIC READING (LOG TRANSMISSION)	EXPECTED READING	ERROR
(ML/50 MLS)			%
0.125 (Standard)	0.080	(0.080)	0.0
0.250	0.160	0.160	0.0
0.500	0.320	0.320	0.0
0.750	0.465	0.480	3.2
1.000	0.615	0.640	3.9
1.500	0.940	0.960	2.4





Results

Readings

0.5 mg. phenothiazine alone	0.330 $\pm$ 0.5 mg. Recovery (100%)
0.5 mg. phenothiazine and 0.5 mg. thionol	0.468 $\pm$ 0.709 mg. Recovery (142%)
0.5 mg. phenothiazine and 0.5 mg. phenothiazone	0.532 $\pm$ 0.806 mg. Recovery (161%)
0.5 mg. phenothiazine and 0.5 mg. phenothiazine-sulphoxide	0.340 $\pm$ 0.515 mg. Recovery (103%)

therefore each of these derivatives, particularly phenothiazone and thionol, interfere with the colorimetric estimation of phenothiazine.

Recoveries

Weighed amounts of phenothiazine were added to biological material and estimated. Soxhlet extractions were carried out for varying times (see Table IV) and showed that extending the times of extraction beyond twenty-four hours did not improve recoveries. The twenty-four hour extraction period was therefore adopted for subsequent determinations. The average recovery obtained in eight twenty-four hour extractions was 87.2% (Range 77.8 - 100%).

In view of the relatively low recoveries obtained, the method used by Collier (1940 b) was tested. This involved drying the material by heating at 110°C and triple extraction with boiling 95% alcohol, followed by bromination and colorimetric determination. The results of these experiments are given in Table V.

TABLE IV

The effect of duration of extraction on the recovery of Phenothiazine  
added to 10 g. colour contents

<u>Hours Extracted</u>	<u>No. of observations</u>	<u>PT added (mg)</u>	<u>Recovery % or Mean Recovery %</u>	<u>Standard error or Range</u>
3	2	50	60.9	R. 60.6 - 61.3
3	1	50	77.3	
7.5	1	20	57.0	R. 82.9 - 88.4 S.E. 1.47
14	1	50	77.3	
18	1	50	69.3	
19	3	50	86.2	
24	8	50	87.2	
28	1	50	71.8	
40	1	50	86.4	
48	1	50	77.2	

**TABLE V.**

The Recovery of added Phenothiazine from 10g. colon contents, using Collier's method

Added PT (mg.)	Estimated PT (mg.)	Recovery (%)
5.0	6.35	127
5.0	8.25	165
50.0	47.75	95.5
50.0	88.0	176
50.0	50.0	100
50.0 •	53.5	107
		Mean recovery = 123.4%

• 1 g. colon contents used

A possible explanation for the higher recoveries (mean 128%) obtained with Collier's method might be the formation of coloured oxidation products of phenothiazine. To test this hypothesis 50 mg. batches of pure phenothiazine were mixed with samples of colon contents, dried at  $110^{\circ}\text{C}$  and extracted with various hot solvents, each of which was known to dissolve one or more phenothiazine derivatives (see solubility tables, pages 36, 37 and 38). These solvents were water, acetone, ether, alcohol, 95% alcohol, benzene, xylol, and toluene. (A control sample of colon contents without added phenothiazine was similarly treated, as various pigments in this material were also soluble in these organic liquids). The resulting extracts were subjected to separation by paper chromatography. Apart from the zones of colour caused by normal colon contents, the chromatograms showed only the presence of phenothiazine. The high readings obtained after heating to  $110^{\circ}\text{C}$  could not therefore be attributed to the formation of known coloured oxidation products.

Recovery from faeces of phenothiazine-dosed sheep

Only phenothiazine was present in petroleum ether and hot water extracts of the faeces of these sheep. The amounts of phenothiazine recovered from the faeces of five sheep for four to five days after dosing with 190 - 320 mg/kg phenothiazine are given in Table VI. The recoveries ranged from 8.37% to 44.2%. From two of the sheep the recovery was about /



about 44% while for another two sheep it was approximately 16%. Only 8% was recovered from the remaining sheep. The reason for these unusual results was unknown.

Recovery from urine and faeces of sheep dosed with phenothiazine derivatives.

One sheep was dosed with each of the following derivatives, phenothiazone, thionol and phenothiazine-sulphoxide. The results are shown in Table VII.

Of the phenothiazone, 21.7% was recovered unchanged in the faeces, with traces of the same compound in the urine. A small quantity of an unknown liquid substance was also obtained. When thionol was administered, 29% was recovered unaltered in the faeces and 4% in the urine in company with an unknown compound. After phenothiazine-sulphoxide was given by mouth, 5% was recovered as phenothiazine in the faeces, while 0.4% was estimated in urine as phenothiazine and phenothiazone. Only traces appeared unchanged in the faeces. Recoveries of phenothiazine added to urine and steam distilled showed an average of 61%. (Range 45 - 87%).

Recovery from urine and faeces of rabbits dosed with phenothiazine

Paper chromatograms of petroleum ether, ether and chloroform extracts of urine and faeces of phenothiazine-dosed rabbits showed that the urine contained phenothiazine and phenothiazone while the faeces contained phenothiazine only.

Using /

TABLE VI

Recovery of Phenothiazine from faeces of sheep for 4-5 days  
after dosing with Phenothiazine

SHEEP	DOSE (mg/kg.)	RECOVERY %
1 *	192	8.37
1 *	316	15.2
2	288	16.05
3	265	44.2
4	269	43.3
		(Mean 25.42)

\* Same individual

TABLE VII

Recoveries from faeces and urine of sheep dosed with Phenothiazine derivatives

	DOSE (mg/kg.)	FAECES	URINE	TOTAL	No. of days before excretion stopped
Phenothiazine	115	PO 21.76% trace PT.	Trace PO and trace liquid	21.76%	4
Thionol	115	Th. 29.3%	Th. 3.8% and trace unknown	33.1%	5
Phenothiazine-sulphoxide	258	PT 5.3% traces PSO and PO	PT and PO 0.42%	5.7%	4

PT = Phenothiazine      PO = Phenothiazine  
 PSO = Phenothiazine-sulphoxide      Th = Thionol

Using the steam distillation method average recoveries of 20.8% (Range 16.8% - 23.4%) phenothiazine (Table VIII) and 23.5% (Range 16.2% - 30.2%) phenothiazone (Table IX) were obtained in the urine of dosed rabbits.

The recovery of phenothiazine from the faeces of five rabbits is given in Table VIII. The mean recovery was 1.9% (Range 0.4% - 4.7%). The mean total recovery of both compounds from the urine and faeces of the rabbits was 46.3% (Range 43.8% - 50.2%). A higher recovery of phenothiazine in the faeces and less phenothiazone in the urine was obtained from the only rabbit which was fed on greens during the experiment. Faeces and urine from animals given propylene glycol were negative when tested for phenothiazine and derivatives.

Recoveries from rabbit liver, kidneys and bile juice

Extracts of the liver and kidneys of a rabbit which was dosed with phenothiazine and killed eighteen hours later showed traces of phenothiazine, but none of the known oxidation products. Examination of extracts of the bile juice showed that a small quantity of phenothiazone and traces of phenothiazine-sulphoxide were present.



TABLE VIII

Recovery of Phenothiazine from urine and faeces of rabbits for  
4-5 days after dosing with Phenothiazine

Rabbit	Dose (mg/Kg.)	Recovery %		
		Urine	Faeces	Total
(1)	165	19.9	1.0	20.8
(2)	253	22.8	1.9	24.7
(3) <sup>a</sup>	307	23.4	4.7	28.1
(4)	354	16.8	1.7	18.5
(5)	580	20.9	0.4	21.3
		Mean	Mean	Mean
		20.8	1.9	22.7

<sup>a</sup> Rabbit fed on greens

TABLE IX

Recovery of Phenothiazone from urine of rabbits for 4-5 days  
after dosing with Phenothiazine

Rabbit	Dose	Recovery %	Total Recovery
	mg/Kg. Phenothiazine	Phenothiazone	Phenothiazine and Phenothiazone (Urine and Faeces). %
(1)	165	23.4	44.1
(2)	253	20.3	44.9
(3) •	307	16.2	43.8
(4)	354	30.2	48.7
(5)	380	27.5	50.2
		Mean 23.5	Mean 46.3

• Rabbit fed on greens

Recoveries from mice.

Considerable difficulties were encountered in the intubation and administration of the drug to these animals. The results of these experiments are given in Table X.

TABLE X

Findings after mice had been dosed with Phenothiazine

No. of mice	Dose.	URINE			FAECES			LIVER			KIDNEYS			REST OF BODY		
		PT	PO	Th	PT	PO	Th	PT	PO	Th	PT	PO	Th	PT	PO	Th
4	10 mg. (approx.)	x	x	trace	x	trace	-	no traces			found			no traces found		
3	10 mg.	0.8	x	trace	0.54	trace	-	"	"	"	"	"	"	"	"	"
		mg. (mean)			mg. (mean)											
5	100 mg.	not examined			not examined			"	"	"	"	"	"	"	"	"

PT - Phenothiazine  
PO - Phenothiazone  
Th - Thionol

In the control animals the results of all tests were negative.

Results of Incubations

The paper chromatograms made from the extracts obtained after phenothiazine was incubated with sheep rumen contents showed only traces of phenothiazone in addition to phenothiazine. Only in some incubation experiments was phenothiazone found. The phenothiazine after incubation appeared as two concentric circular zones. A little of the phenothiazine appeared in its /

its normal position at the solvent front when 8:1 petroleum ether: acetone was used as the developing solution. More of it appeared in a zone further back. Only phenothiazine was found in extracts after incubation of phenothiazine with horse dorsal colon contents.



## Discussion of Results

### Recoveries in the faeces of dosed sheep

Only phenothiazine was found in the sheep faeces. This confirmed the findings of Collier (1940 b) and Lipson (1940).

The quantitative discrepancies in the results of previous workers who have estimated phenothiazine in the excreta may be explained in part by the inadequacies of their methods of estimation.

In the experiments described in this thesis the accuracy of the colorimetric readings of brominated solutions of pure phenothiazine (Table III, page 61) agreed with that reported by Cupples (1942). The trial recoveries showed an average of 87%. The actual recoveries from the faeces of treated sheep showed a wide scatter. (Table VI, page 68). The reason for this variation was not clear, as exactly the same method was employed in each case, the sheep were of the same age, sex and breed, and were kept under the same conditions. Apparent discrepancies between the results of different workers may be due to this variation. The variation in the proportion absorbed may provide an explanation for the fact that some host animals show severe toxic effects after therapeutic doses of the drug, while others /

others, even after enormous doses, are apparently unaffected. Recoveries obtained in this work when using Collier's method were high. (Table V, page 65). Swales and Collier (1940) recovered about 40% of the dose in the faeces, although they also found the amount to vary widely in different individuals. Lipson and Gordon (1940) with twelve sheep recovered an average of 32% in the faeces; the range was 15 - 63%. More recently Harpur, Swales and Denstedt (1950) found just over 20% in the faeces of dosed sheep. Collier, Allen and Swales (1943) showed that the particle size of the phenothiazine preparation could affect the proportion absorbed. After the administration of micronised phenothiazine (particles under  $4 \mu$  in diameter) only 10% was recovered from the faeces, while with ordinary commercial forms 28 - 36% was excreted in the faeces.

#### Recoveries from Urine of Dosed Sheep

It was unfortunate that no quantitative estimations were made on sheep urine in these experiments. At that time no method had been found to estimate the compounds present in urine. However, it is apparent from the results of other workers that a conjugate of leuco-phenothiazone, probably potassium leuco-phenothiazone sulphate is the chief excretory product in the urine of treated sheep. Collier, Allen and Swales (1943) also found traces of leucothionol. Clare (1947) found these two compounds, and in addition unconjugated leucophenothiazone, and /

and phenothiazine as an unknown conjugate from which it could be released by acidification. This phenothiazine conjugate was also described by Lipson (1940). Alexander, Mackie, Ghatge and Waddell (1958) found phenothiazine, phenothiazone and phenothiazine-sulphoxide in the urine of phenothiazine-dosed sheep.

#### Quantitative recoveries in sheep urine

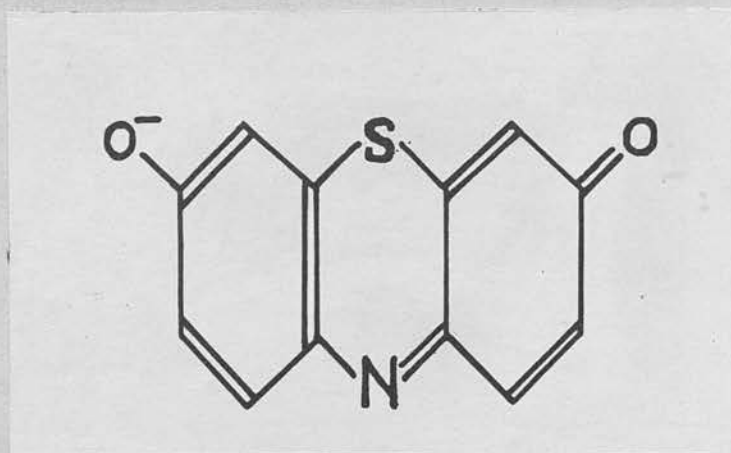
Swales and Collier (1940) obtained 40 - 50% of the dose in urine as derivatives and Harpur, Swales and Denstedt (1950) recovered 40%. Collier, Allen and Swales (1943) showed that when micronised phenothiazine was given, 55 - 96% of the dose was excreted in urine while 46 - 65% of commercial forms was excreted in urine.

#### Recovery of phenothiazine derivatives in faeces and urine of sheep dosed with phenothiazine derivatives

Results obtained when sheep were dosed with phenothiazine derivatives are of interest because no detailed study of the excretion of these compounds has been made before. De Eds and Thomas (1941) gave thionol to rabbits and afterwards identified the same compound in the bile and faeces. Collier, Allen and Swales (1943) gave 5g. phenothiazone to a sheep and recovered phenothiazone unchanged from the faeces and from the urine, phenothiazone in both free and conjugated forms.

Similar /

Similar but qualitative results to those in Table VII were obtained from the urine of sheep dosed with derivatives by Alexander, Mackie, Ghatge and Waddell, (1958). The methods in this case were quite different. No thionol was then reported in the urine of thionol-dosed sheep, but the blue compound mentioned as having similar chemical properties to thionol but a different absorption spectrum, may in fact have been thionol, as the spectrum has been shown to vary considerably when different degrees of ionisation exist, i.e. in different solvents or at different pH values. (Granick and Michaelis, 1947). The spectrum then obtained is not significantly different from that of thionol when in the form depicted.



This is obtained in 0.01N NaOH solution. (Granick and Michaelis, 1947).

In /



In view of the fact that Collier, Allen and Swales (1943) found that dosed phenothiazone was quickly and almost completely absorbed high in the alimentary tract, and that it is rapidly re-absorbed after excretion in the bile, (Harpur, Swales and Denstedt, 1950), it is unexpected that as much as 22% of the dosed phenothiazone was excreted unchanged in the faeces, while only traces were found in the urine.

#### Rabbits

Only phenothiazine was found in rabbit faeces after dosing with phenothiazine. De Eds and Thomas (1941) found phenothiazine and leucothionol in rabbit faeces.

In rabbits the recovery of phenothiazine from the faeces ranged from about 0.5% - 5%. (Table VIII, page 71). The doses given to the rabbits, although relatively higher than those administered to the sheep, were fairly low when compared with those given by other workers. However, in a single experiment a rabbit was given a larger dose of phenothiazine (2,110 mg/kg) and as much as 20% was recovered from the faeces. This indicates that there may be a limit to the amount which can be absorbed. Of the five rabbits given smaller doses, one which was fed on greens throughout the experiment had most in the faeces. The influence of diet on the fate of phenothiazine has been mentioned by Harpur, Swales and Denstedt (1950) and would be an interesting study in itself. Changes in diet are known /

known to alter the flora of the digestive tract and its pH. They may also influence the rate of movement of intestinal contents. (Read, 1950).

The recoveries from the urine of dosed rabbits cannot be considered really quantitative, because trial recoveries averaged 61% and their range was high. The figures obtained for the percentage recoveries of phenothiazine and phenothiazone are given. (Tables VIII and IX, pages 71 and 72). Qualitatively both phenothiazine and phenothiazone were found, thionol was only identified in the urine when a larger dose had been given. A slightly greater percentage of phenothiazone than phenothiazine was recovered in the urine in most cases. An exception was in the case of the rabbit which had been fed on greens. In this instance a greater percentage of phenothiazine than of phenothiazone was recovered from the urine. Recoveries in the urine of the bran-and-oats-fed rabbits were quite constant, indicating that in non-ruminants absorption may be less labile than in ruminants.

De Eds, Eddy and Thomas (1938) had found conjugated phenothiazine and a compound which they at first thought to be leucothionol, both free and in a loose chemical combination in the urine of dosed rabbits. The phenothiazine was present as a conjugate which could be hydrolysed by acid in the cold and was identified by its melting-point and mixed melting-point. (De Eds, Eddy and Thomas, 1938). Their identification of the system /

system they thought to be thionol-leucothionol was based on the comparison of the potentiometric characteristics of the oxidation-reduction system with those of known systems. Later the compound released from the loose combination was shown to be leucophenothiazone by its melting-point. (De Ede and Thomas, 1942).

Collier, Allen and Swales (1943) found mainly leucothionol with little leucophenothiazone in rabbit urine.

Alexander, Mackie, Ghatge and Waddell (1958) found phenothiazine, phenothiazone and a red solid, melting-point  $208^{\circ}\text{C}$ , in the urine of phenothiazine-dosed rabbits.

Less than 50% of the dose has been recovered from the excreta of treated horses. (Swales, Collier and Allen, 1942). Most of this was in the form of leucophenothiazone.

The urine of phenothiazine-dosed pigs contained mostly conjugated phenothiazine, with small amounts of leucothionol, and traces of unconjugated leucophenothiazone. (Collier, Allen and Swales, 1943).

Clearly the excretion of orally administered phenothiazine varies considerably in different animals, so that results from one species cannot be expected to apply to another.

Recoveries in general appear to be very incomplete, so that it is quite possible that part of the dose is altered in /

in some way to an unknown but active compound. Ellison and Todd (1957) and others have shown that neither phenothiazine nor its derivatives are retained in the body for more than one hundred and twenty hours. But Taylor and Sanderson (1940) fed faeces from a phenothiazine-dosed goat to an infected goat, and obtained no anthelmintic effect, which implies that the active metabolite, if it exists, may be absorbed low in the alimentary tract.

#### Recovery in rabbit bile

Bile from the rabbit that was killed after phenothiazine dosage contained phenothiazone and phenothiazine-sulphoxide. This agreed with the results of Clare in sheep and calves (1947) while Harpur, Swales and Denstedt (1950) found only phenothiazone in the bile of these animals. Harpur et al. (1950) found that after phenothiazone was excreted in the bile it was re-absorbed before the caecum was reached.

#### Incubation Experiments

After phenothiazine had been incubated with sheep rumen contents a trace of phenothiazone was found, although no derivatives were found after horse colon contents were incubated with phenothiazine. Harpur, Swales and Denstedt (1950) had incubated phenothiazine with sheep rumen contents and obtained no evidence that oxidation had taken place. However their methods /



methods may have been too insensitive to detect minute quantities of the derivatives. Harpur et al. (1950) found only unchanged phenothiazine in the rumen of dosed sheep, although Collier, Allen and Swales (1943) claimed to have found phenothiazone in the rumen after the administration of 100 g. phenothiazine. Clare (1947) found no phenothiazone in the alimentary tract except in the caecum but identified phenothiazine-sulphoxide in the ruminal, abomasal and intestinal contents. Esserman (1952) found no derivatives after incubating phenothiazine with intestinal contents from rats and chicks, or on analysis of intestinal fluids from dosed animals.

It was interesting that the phenothiazine extracted from the incubation flasks ran on paper to form two distinct circular zones (pages 73-74). Esserman (1952) has described the formation of a phenothiazine-fat complex in the intestine of dosed rats and chicks. This complex "trailed" on the chromatogram in a similar manner. She was unable to produce this complex under anaerobic conditions in vitro.

Because of these conflicting results it is obviously not possible at present to conclude whether phenothiazine is oxidised before or after absorption in the alimentary tract. Some, at least, of it must be absorbed unchanged because it is excreted as a conjugate in the urine. Harpur, Denstedt and Swales /

Swales (1950) found phenothiazine in the blood draining the rumen of phenothiazine-dosed sheep, whereas Clare (1947) found phenothiazine-sulphoxide in the portal blood. Both groups of workers agreed, however, that only leucophenothiazone was present in the systemic circulation of normal sheep after treatment.

Harpur, Denstedt and Swales, (1950), although they admitted the possibility of phenothiazine-sulphoxide being formed during rumination, in contact with alkaline saliva, concluded that phenothiazine was absorbed as such, high in the alimentary tract, and was oxidised afterwards. Clare (1947) concluded that phenothiazine was oxidised to phenothiazine-sulphoxide in the alimentary tract and then absorbed into the portal circulation. In normal sheep he postulated that absorbed phenothiazine-sulphoxide was completely converted in the liver to leucophenothiazone and then conjugated to form potassium leucophenothiazone sulphate. In old or debilitated sheep or those given excessive doses of phenothiazine, he found conversion to leucophenothiazone to be incomplete. This was revealed by the appearance of phenothiazine-sulphoxide in the systemic circulation.

The fact that phenothiazone given orally was without anthelmintic effect (Collier, Allen and Swales, 1943) does not entirely /

entirely rule out the possibility that this derivative may be the active metabolite of phenothiazine. The same workers administered 23g. phenothiazone to a sheep which died eight hours later and obtained evidence that it was very quickly absorbed or altered; only 5g. remained in the rumen, and traces in the rest of the alimentary tract. It seems possible that when phenothiazone itself was administered, very little of it actually came in contact with the worms. The less soluble phenothiazine is likely to be more slowly absorbed and may be oxidised slowly, releasing phenothiazone in its passage down the alimentary tract.

The recovery of as much as 22% of phenothiazone from the faeces of phenothiazone-dosed sheep (Table VII, page 69) does not, however, lend support to this view.

The discovery by Esserman, (1952) using chromatography and microionophoresis, that worms expelled after treatment of the host with phenothiazine contained only phenothiazine and no known oxidation products, seems to provide conclusive evidence that these compounds are not responsible for the anthelmintic action of phenothiazine.

### General Discussion

One of the factors which is known to influence the anthelmintic efficiency of phenothiazine is that of the particle size of the preparation used. (Gordon, 1956). Commercial grades of phenothiazine are generally fairly coarsely ground because the production of a powder containing fine particles in quantity is expensive. It is remarkable that although a preparation of phenothiazine consisting of particles of under 4  $\mu$  has been shown to be more completely absorbed from the alimentary tract than ordinary commercial forms, (Collier, Allen and Swales, 1943) its anthelmintic action is enhanced (Gordon, 1956) and not reduced, as one would imagine.

Gordon (1956) has shown that the success against some species of worms was less influenced by the particle size than others, although in general the efficiency was reduced when the particle size was greater than 20  $\mu$ . Whitten (1956) in an attempt to account for the varying success of phenothiazine against Trichostrongylus spp. tested preparations of different particle sizes against T. colubriformis in sheep. He found that preparations containing a majority of particles smaller than 10  $\mu$  were markedly superior to coarser preparations. His results have since been confirmed by Kingsbury (1958).

Thomas /



Thomas and Elliot (1957) found that a preparation with a mean particle size of 1  $\mu$  compared with one of 10  $\mu$  increased the efficiency of phenothiazine against Ostertagia spp., Trichostrongylus colubriformis, and T. axei, made little difference against Haemonchus contortus and Cooperia spp., and decreased the effect against the worms of the lower bowel, Oesophagostomum venulosum and Chabertia ovina. They conjectured that the finer particles may have been absorbed before reaching the lower bowel, and suggested that an ideal preparation should consist of a large number of particles below 5  $\mu$  with a smaller number from 5  $\mu$  - 30  $\mu$ .

Phenothiazine, like most other anthelmintics, is selective in its action against nematodes. (Griffiths, 1954). It is perhaps best known for its success against the nematodes of sheep yet it is relatively ineffective against Strongyloides, Nematodirus, and Trichostrongylus spp., in the small intestine, and against Trichuris in the caecum. It is also inactive against lungworms, possibly because it does not reach them in an active form. In both sheep and cattle it has little effect on Chabertia ovina, in the colon. Large doses are required against most parasites but Haemonchus contortus of the abomasum requires less than the rest. Phenothiazine is quite ineffective as an anthelmintic in carnivores. If the reason /

reason for this selective action could be found it might provide a valuable key to the mode of action of the drug.

It may be selective because the drug or its active metabolite may be absorbed by the host or altered and so prevented from reaching certain nematodes in the alimentary tract. The varying rate of movement of digesta in different parts of the alimentary tract may mean that some parasites are exposed to the drug for a shorter time than others. Read (1950) has suggested that varying pH values in different parts of the alimentary canal may influence the ionisation and thus the properties of various drugs. In sheep and cattle the nematodes of the small intestine seem to be less affected by phenothiazine than the majority of the others, while it is most effective against those of the abomasum.

Ellison and Todd (1957) reported that only very small amounts of phenothiazine were found in the small intestine of dosed calves, and that the chief sites of concentration were the rumen, abomasum and colon. In horses and other monogastric herbivores, the most effective anthelmintic activity of phenothiazine seems to be on nematodes in and distal to the caecum. Yet the rate of movement of digesta through the abomasum and small and large intestine of sheep is rapid when compared with the rate of movement through the colon and caecum of the horse. (Read, 1950).  
There /

There is therefore some evidence that the low efficiency of phenothiazine against worms of the small intestine of ruminants may be a result of the low concentration of the drug found in this organ. Similar explanations do not appear to account for the relative efficiency of the drug against worms in different host species.

Another possible explanation for the selectivity of phenothiazine is that only certain parasites are able to take up or absorb the drug.

Davey and Innes (1942) had concluded from the evidence then available that the nematode cuticle was impermeable to phenothiazine and that particles of the drug had to be taken in through the mouth of the worm. They considered that a large dose of phenothiazine was necessary for anthelmintic activity because a certain concentration of particles had to be present for the nematodes to take in adequate amounts by mouth. However, Lazarus and Rogers (1950) using phenothiazine labelled with sulphur-35 demonstrated that the nematode cuticle was permeable to phenothiazine by showing that the rate of uptake of the drug by male Ascaridia galli in vitro was hardly affected when all the orifices were ligatured.

The work with labelled phenothiazine has produced many interesting results but the technique has the disadvantage that /



that it is impossible to distinguish between phenothiazine and its sulphur-containing derivatives.

Lazarus and Rogers (1950) measured the rate of uptake of phenothiazine in three types of nematodes, Haemonchus contortus from sheep, Ascaridia galli from poultry and Nippostrongylus muris from rodents (examples respectively of extremely phenothiazine-sensitive, fairly sensitive and resistant parasites). They found that Haemonchus contortus took up phenothiazine more rapidly in vitro than Ascaridia galli and Nippostrongylus muris. This result lends support to the theory that the relative permeability of the cuticle of the nematode to the drug may account for the degree of sensitivity shown by that species. Yet Esserman (1952) found that there was no appreciable difference in uptake of the drug between Ascaridia galli and Nippostrongylus muris when their respective hosts were dosed with phenothiazine, although the Ascaridia galli were expelled, and the Nippostrongylus muris apparently unaffected by this treatment. Esserman (1952) found that the phenothiazine-fat complex she had described penetrated into Ascaridia galli more slowly than pure phenothiazine in vitro.

Lazarus and Rogers (1951) showed that Haemonchus contortus, Ascaridia galli and Nippostrongylus muris took up phenothiazine from the medium four to ten times more quickly in vivo than in vitro and that the rate of uptake could be increased /



increased by the presence of wetting agents. They pointed out, however, that in the case of nematodes like Ascaridia galli which may feed on intestinal contents, the parasites do not feed on the medium in vitro and that it is quite possible that some of the drug is taken in by mouth in vivo. (Rogers and Lazarus, 1949a).

Trim (1949) studied the cuticle of Ascaris lumbricoides var suis and found that it consisted mainly of protein, with a very small amount of lipid material, probably spread as a thin film over the surface of the worm. Later, Bird (1956) using Ascaris lumbricoides and Strongylus equinus was able to show up the lipid film with Sudan Black. He found small amounts of carbohydrate in the cuticle, and showed that the amino acid content of the cuticle protein varied slightly in different species. The penetration of many substances into Ascaris appeared to depend on their being partitioned between the medium and the lipid layer on the cuticle. (Trim, 1949).

The evidence available shows that phenothiazine is taken up by nematodes which are resistant to its actions, as well as those which are sensitive to the drug.

Phenothiazine may produce its anthelmintic activity by interfering with a metabolic process only found in susceptible parasites. On the whole this may provide the most probable explanation for its selectivity. Phenothiazine produces quite different /

different reactions in different mammals which have on the whole, a similar metabolism, and therefore it would not be surprising to find such a variation in response between parasitic nematodes, which are thought to vary considerably in their fundamental metabolic processes. (Bueding, 1949).

Lazarus and Rogers (1951) pointed out that phenothiazine is active against worms which eat ingesta (e.g. Ascaris lumbricoides) and those which feed on host tissues (Strongylus spp.). Most of the nematodes which are sensitive to the drug are blood-suckers; it is not certain whether these have a fundamentally different metabolism from other nematodes. Differences in metabolism between host and parasite may not be necessary for differences in toxicity, as the drug may never reach toxic concentration in the host. (Lazarus and Rogers, 1951).

Because the oxygen tension is low in the alimentary tract, metabolic processes of nematodes are likely to be chiefly anaerobic. (Bueding, 1949). However, in certain nematodes, e.g. Nippostrongylus muris, Nematodirus spp., and Haemonchus contortus, aerobic mechanisms appear to play an important role. (Bueding, 1949).

The oxygen tension of the alimentary tract has been shown to be lower in ruminants (Read, 1950) and higher in pigs (Bueding, 1949) than in most domestic animals. Some nematodes, especially /

especially those living near the mucosa, may have access to more oxygen than would be expected by analysis of the intestinal contents, because oxygen may diffuse from the mucosa into the lumen. (Read, 1950).

As the drug has its greatest effect on nematodes of ruminants and relatively little on those of pigs several authors have sought for correlations between these facts.

The inhibition of hexokinase by phenothiazine, which has been mentioned as having possible significance (Allenby and Collier, 1952) would be expected to have a more profound effect on the "anaerobic" helminths, i.e. those which obtain their energy through glycolysis. Nematodes of pigs, having access to more oxygen than those of ruminants, would be less dependant on this metabolic process.

Lazarus and Rogers (1951) put forward a tentative hypothesis that Nippostrongylus muris was not affected by phenothiazine because this species does not depend on energy from anaerobic sources. Haemonchus contortus, however, with a relatively aerobic metabolism, is probably the most sensitive nematode to the action of phenothiazine.

A possible alternative explanation for the selectivity shown by phenothiazine is that the drug may be altered in the alimentary tract to a compound or compounds which may be only /

only available to some nematodes. If this change does not take place in certain hosts, e.g. carnivores, this would explain why the compound is ineffective in these animals. If it is only formed in certain parts of the alimentary tract in other animals this would explain some of its selective action against different nematodes in one species of host.

Probably the most promising field of investigation for the future study of anthelmintics lies in the study of the normal metabolism of nematodes. At present, knowledge of their physiological processes is very incomplete. Without this knowledge it is not possible to ascertain the mode of action of any particular anthelmintic.



### Conclusion

Even when taking into account the inadequacies of the methods for quantitative estimation used, it is clear that a considerable proportion of a dose of phenothiazine remains unaccounted for in the excreta. As it has been shown that the compound and its known metabolites are not retained in the body for longer than five days, the explanation for the low recoveries may be that unknown derivatives are formed in the body. If this is the case these substances may prove to be the anthelminthic substances.

This theory provides a possible explanation for the large quantity of phenothiazine required for anthelminthic activity. If the unknown derivative or derivatives were only formed in certain parts of the alimentary canal, or in some species of host, this theory would also explain why the drug is selective in its action.

SUMMARY

1. Attempts were made to keep nematodes in vitro in an incubator, and in nylon bags inside the lumen of the horse intestine. These were mostly unsuccessful.
2. Phenothiazine and its oxidation products were separated by paper chromatography.
3. Methods for estimating Phenothiazine in biological material were devised, giving mean recoveries of 87.2% (S.E. 1.47) in faeces and other semi-solids and 61.6% (Range 45-87%) in urine. Methods were also devised for the estimation of phenothiazine derivatives in faeces and urine.
4. A mean of 25% (Range 8% to 44%) of the total amount of phenothiazine given to sheep was recovered in the faeces in four to five days.
5. Individual sheep were dosed with thionol, phenothiazone and phenothiazine-sulphoxide. Of the thionol, 32.7% was recovered, of the phenothiazone 21.8%, and of the phenothiazine-sulphoxide 5.7%, mostly as phenothiazine.
6. A mean of 22.7% (S.E. 1.67) phenothiazine and 23.5% phenothiazone was recovered from the excreta of phenothiazine-dosed rabbits. The mean total recovery was 46.2%.
7. /

7.           Traces of phenothiazine but no trace of oxidation products were found in extracts of liver and kidneys of a phenothiazine-dosed rabbit. The bile juice contained phenothiazone and phenothiazine-sulphoxide.
8.           Phenothiazine was incubated with sheep rumen contents, often resulting in the formation of traces of phenothiazone.

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